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TITLE: Targeting Androgen Receptor in Breast Cancer: Enzalutamide as a Novel Breast Cancer Therapeutic

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14. ABSTRACT In breast cancers, the androgen receptor (AR) is more widely expressed than estrogen receptor alpha (ER) or the progesterone receptor (PR) (1), which are used as therapeutic targets and biomarkers, suggesting a potential role for AR in BC. To explore the function of AR in models of the three main subtypes of breast cancer (ER positive, ER negative and Her2+), we are using a new-generation AR inhibitor, enzalutamide, which impairs nuclear localization of AR. This is a very different mode of action than previous generation anti-androgens such as bicalutamide (Casodex), which is a competitive inhibitor of endogenous androgens that allows ligand-mediated nuclear localization of AR. Enzalutamide has shown success in the clinic in patients with late stage prostate cancer. The research in this proposal seeks to determine whether inhibition of AR with enzalutamide will be effective in breast cancer and utilize preclinical models of the three main subtypes of breast cancer to determine if and how it should be combined with currently used standard of care treatments in the three main types of breast cancer, with the primary objectives of the research being to guide the design of future clinical trials with enzalutamide.					
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INTRODUCTION:

In breast cancers, the androgen receptor (AR) is more widely expressed than estrogen receptor alpha (ER) or the progesterone receptor (PR), which are used as therapeutic targets and biomarkers, suggesting a potential role for AR in BC. We examined the primary tumors of women treated with tamoxifen or aromatase inhibitor therapy and found that a higher AR to ER protein ratio correlates with worse response to traditional the anti-estrogen tamoxifen (see figure 1 in our published manuscript Cochrane et al in the appendix). To explore the function of AR in models of the three main subtypes of breast cancer (ER positive, ER negative and Her2+), we are using a new-generation AR inhibitor, enzalutamide, which impairs nuclear localization of AR. This is a very different mode of action than previous generation anti-androgens such as bicalutamide (Casodex), which is a competitive inhibitor of endogenous androgens that allows ligand-mediated nuclear localization of AR. Enzalutamide has shown success in the clinic in patients with late stage prostate cancer refractory to bicalutamide and is now FDA approved as a prostate cancer therapy. The research in this proposal seeks to determine whether inhibition of AR with enzalutamide will be effective in breast cancer and utilize preclinical models to determine if and how it should be combined with currently used standard of care treatments in the three main types of breast cancer, with the primary objectives of the research being to guide the design of future clinical trials with enzalutamide.

KEYWORDS: Breast cancer, androgen receptor, estrogen receptor, growth factors, enzalutamide, endocrine resistance, targeted therapy

ACCOMPLISHMENTS: Below we describe for each task in the official statement of work the major activities; specific objectives; significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or other achievements. We include a discussion of stated goals not met or tasks not fully completed. We include pertinent data and graphs in sufficient detail to explain significant results achieved. Detailed description of the methodology used is provided in the methods section of two manuscripts in the appendix. The first manuscript was published in January of 2014 and was submitted with the first annual progress report. The second manuscript arising from this work investigated AR in the non-LAR subtype of TNBC was published in Jan 2015. We also wrote a review of AR in TNBC. Both of these are listed below and attached. A third manuscript on AR in ER+ breast cancer is almost ready to submit so it is also attached as a pdf at the end of this progress report in the appendix. A fourth manuscript on how androgens expand a cancer stem cell-like population in TNBC and how enzalutamide can best be combined with chemotherapy is in preparation. In preparation for clinical trials in which enza will be tested in combination with chemotherapy, we want to model (with cell lines and TNBC PDX) whether it is better to give it simultaneously or subsequent to chemotherapy, since there is rationale for both approaches.

Barton VN, Gordon MA, Christenson J, D'Amato N, Richer JK. Androgen receptor biology in triple negative breast cancer: A case for AR+ and quadruple negative disease subtypes. *Hormones and Cancer*, 2015 July 23. PMID: 26201402

BartonVN, D'Amato NC, Gordon MA, Lind HT, Spoelstra NS, Babbs B, Heinz RE, Elias A, Jedlicka P, Jacobsen BM, Richer JK. Multiple molecular subtypes of triple negative breast cancer critically rely on androgen receptor and respond to Enzalutamide in vivo. *Mol Cancer Ther*.015 Mar;14(3):769-78. Epub 2015 Feb 23. PMID: 25713333

The objective of Stage I of this proposal is to rapidly generate preclinical data testing enza alone or in combination with standard of care therapeutics in different subtypes of BC to help guide the clinical trials described in **Stage II** (PI clinical partner Dr. Anthony Elias) and steer the rational design and focus on patients most likely to benefit from enzalutamide alone or in combination with currently used therapeutics.

Preclinical Aim 1. To test enzalutamide (enza) in combination with currently approved therapies for breast cancer (BC) in the various subtypes of BC.

Task 1 – Evaluate enzalutamide in combination with anti-estrogen therapy in ER+/AR+ BC lines (MCF7, BCK4) and a ER+/AR+ patient derived xenograft.

Task 2. Test enza in three different tamoxifen resistance models *in vitro*.

Task 3. Test enzalutamide in combination with Her2 directed therapy in ER+ and ER- Her2+ models

Task 4. Examine enzalutamide in combination with an mTOR inhibitor (Afinitor/everolimus)

Task 5. In true TNBC cell lines and explants that retain AR, enzalutamide will be evaluated alone and in combination with chemotherapy and everolimus, *in vitro* and *in vivo*).

What was accomplished under these goals?

TASK1- Evaluate enzalutamide in combination with anti-estrogen therapy in ER+/AR+ BC lines (MCF7, BCK4) and a ER+/AR+ patient derived xenograft. Months 1-4. 100% completed.

We have a manuscript that is almost ready to submit on this topic and the body of the paper and the figures are attached following this report. We will talk about those results later in this progress report.

Below we refer to some data that has not been incorporated into the paper that are directly pertinent to this task. Figure 1 demonstrates that a cell line derived recently from an ER+/AR+ patient derived xenograft (PT12) is stimulated to grow by estradiol (E2) and enzalutamide decreases proliferation in a synergistic manner when enzalutamide is present in a dose dependent manner. We have previously shown in ER+/AR+ MCF7 cells that enza synergizes with both tamoxifen and fulvestrant (see figure 4 of manuscript draft in appendix). However, it is important to show this in a cel line recently derived from a patient as well (Figure 1).

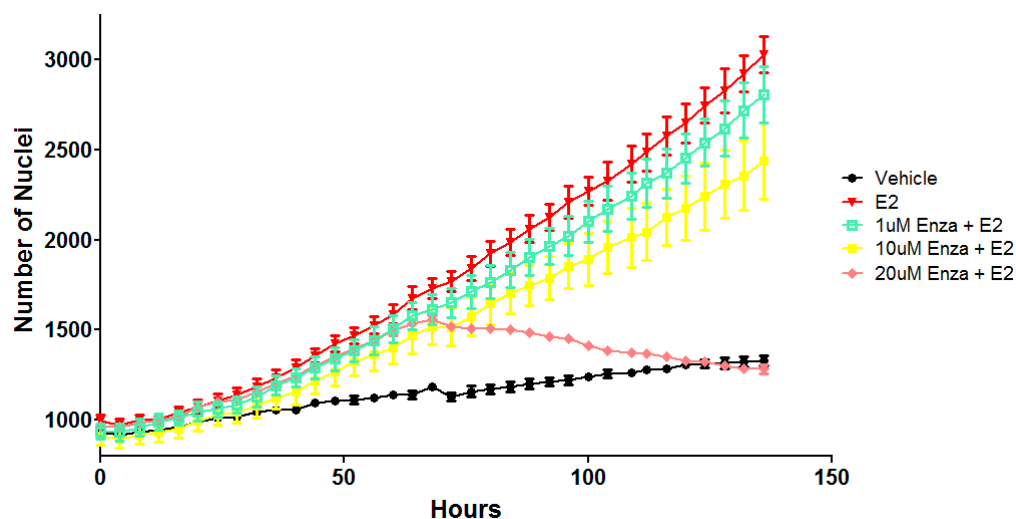
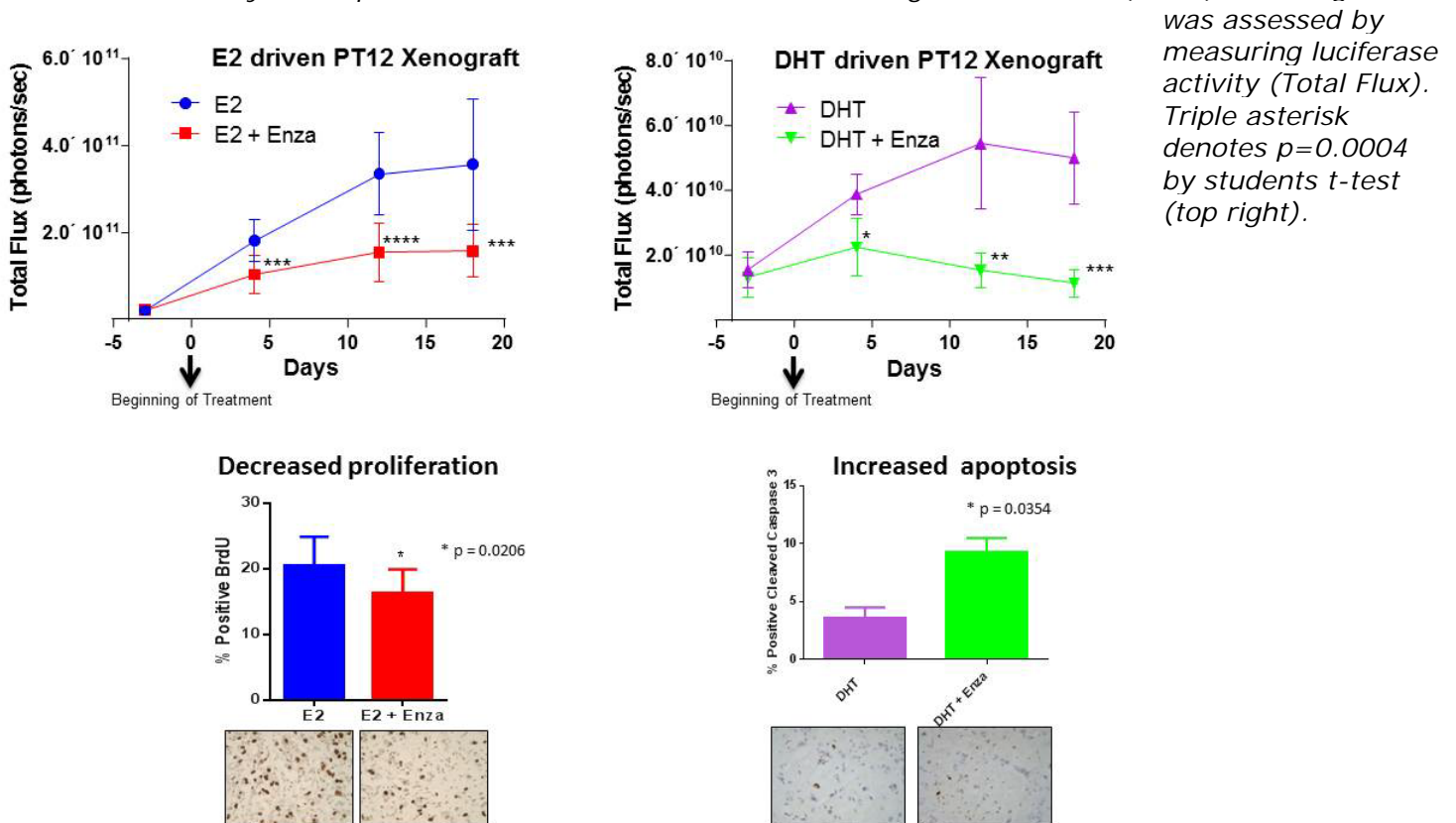


Figure 1: Enzalutamide inhibits estrogen stimulated growth of ER+/AR+ PT12 patient derived tumor cells. 20,000 PT12 cells were plated per well into a 96 well plate in phenol red free and steroid stripped serum and treated with vehicle, estrogen (E2) or E2+Enzalutamide (Enza) at the doses specified. Growth was measured by the IncuCyte live cell imaging system for the specified times.

To determine if enzalutamide would also block both estrogen and androgen-stimulated growth of the PT12 PDX *in vivo*, so the experiment in figure 3 was conducted. Indeed the growth of this AR+ER+ tumor can be stimulated by either estrogen (E2) or androgen, although estrogen does stimulate faster tumor growth than the androgen dihydrotestosterone (DHT). Interestingly, in Figure 2 it can be seen that the primary mechanism for opposing E2-driven tumor growth is a decrease in proliferation (there was not a significant increase in apoptosis). However, the opposite is true for DHT-driven growth, where we observed a significant increase in apoptosis, but there was no decrease in proliferation. We plan on analyzing gene expression in these tumors to

determine exactly what genes are responsible for this difference. We observed the same thing happening with MCF7 xenograft tumors where they grew in response to both E2 and DHT, but the mechanism of decreased tumor growth was different. That was reported by us in last year's progress report and in our Breast Cancer Research manuscript in 2014 (Cochrane DR *et al* 2014). It is interesting and comforting to observe the same thing happening in a patient derived xenograft as we do in the MCF7 cells that have been cultured on plastic for years.

Figure 2: Enzalutamide inhibits estrogen- and androgen-mediated growth of PT12 tumors in different ways. **A)** NOD/SCIDgamma mice implanted with estrogen (E2) pellets of 1.5 mg/per pellet were inoculated with PT12 tumor cells labeled with Luciferase. Mice were randomized at day 0 when the average tumor size was ~ 35mm cubed and placed on control chow or chow containing Enzalutamide (ENZA). Tumor growth was assessed by measuring luciferase activity (Total Flux). Triple asterisk denotes $p=0.0003$ by students *t*-test (top left). Two hours prior to sacrifice, mice with tumors shown in panel "A" were injected with BrdU. Tumors were stained using a BrdU antibody and 10 fields were quantified for BrdU staining for each tumor. Student's *t*-test was used to assess statistical significance (bottom left). **B)** NOD/SCIDgamma mice implanted with dihydrotestosterone (DHT) pellets were inoculated with PT12 tumor cells. Mice were randomized at day 0 when the average tumor size was ~ 16mm cubed. Mice were randomized at day 0 and placed on control chow or chow containing Enzalutamide (ENZA). Tumor growth



The first therapy of choice to treat an ER+ tumor will always likely be an anti-estrogen, even though this may change because we have shown that the relative expression of AR to ER protein (percent cells positive) can predict a poor response to tamoxifen and poor overall survival (Cochrane DR *et al* 2014). We have also shown that the enzalutamide is efficacious in tamoxifen resistant MCF 7 cells *in vivo* (Figure 5 E of manuscript in preparation in appendix). However, ER+ tumors will likely be treated first with tamoxifen (if the woman is premenopausal) or aromatase inhibitor (AI) if post-menopausal or having recurred while on tamoxifen, then if there is a recurrence of disease, with the ER degrader Fulvestrant. Therefore, to model an upcoming company sponsored clinical trial that my partnering PI, Dr. Anthony Elias, is helping with MDV3100-08. An abstract on this trial was accepted to the San Antonio Breast Cancer Conference 2015 in Dec (attached in

appendix), we performed the experiment shown in Figure 3 to test synergy between these two drugs. Since Fulvestrant must be given IM in oil, being able to reduce the effective dose necessary would definitely be helpful. The dose reduction index in figure 3 demonstrates that combining Fulvestrant with Enzalutamide may allow a reduction in the amount of both drugs used to get the same efficacy.

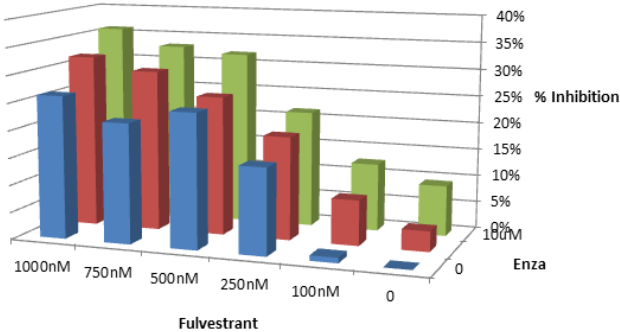
Figure 3. Enza and Fulvestrant synergize to inhibit proliferation of ER+AR+ BCK4 breast cancer cells. Enzalutamide synergizes with Fulvestrant (ICI) to oppose estrogen-induced proliferation of ER+/AR+ BCK4 cells. 20,000 BCK4 cells were plated into 96 well plates in phenol red free media with stripped serum. Cells were treated with increasing doses of Enzalutamide (Enza) and Fulvestrant (ICI) in different combinations for 6 days. Proliferation was measured using the IncuCyte live cell imaging system.

The combination index and dose reduction index were calculated using CalcuSyn.

Enzalutamide		Fulvestrant		Combination Index					
Enza (uM)	% Inhibition	ICI (nM)	% Inhibition	Fulvestrant					
				Enza	100nM	250nM	500nM	750nM	1000nM
5	3.74%	100	0.98%	5uM	0.831	0.652	0.838	1.027	1.238
10	9.29%	250	15.77%	10uM	0.882	0.745	0.768	1.015	1.171
20	14.28%	500	24.58%	20uM	0.835	0.8	0.876	0.938	1.119
		750	21.99%						
		1000	26.23%						

CI < 1 indicates synergy

BCK4 Enza + ICI E2-induced Growth



Dose Reduction Index (Fulvestrant)

		Fulvestrant				
		100nM	250nM	500nM	750nM	1000nM
Enza	5uM	2.873	2.194	1.424	1.093	.880
	10uM	3.916	2.445	1.765	1.216	.998
	20uM	6.121	3.116	1.927	1.522	1.178

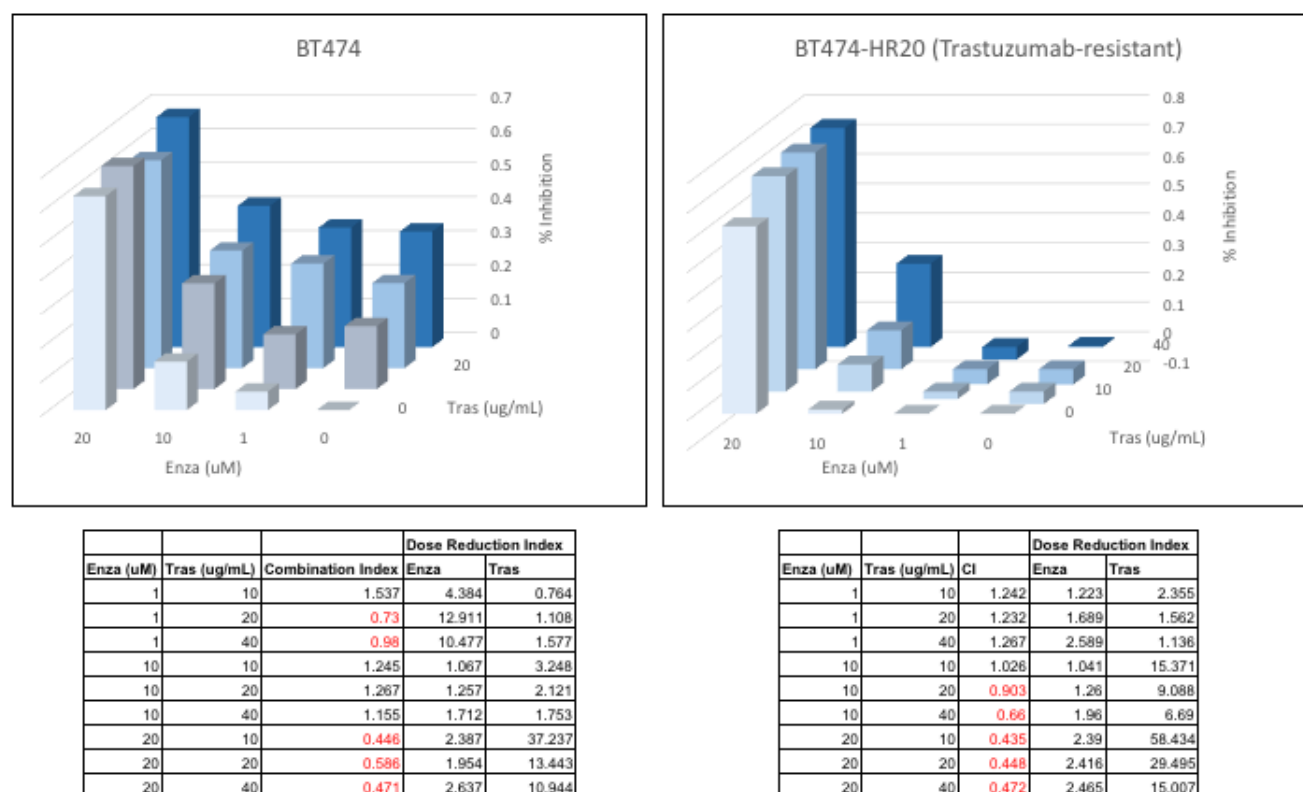
In the paper draft that we have attached to the appendix, we show synergy with enza and tamoxifen or Fulvestrant in additional cell lines.

Task 3. Test enzalutamide in combination with HER2-directed therapy in ER+ and ER- HER2+ models. (90% completed)

In HER2 amplified breast cancer cell lines we have continued the *in vitro* analysis of combining enza with the anti-HER2 agent trastuzumab. We have performed synergy experiments in two related cell lines: the parental BT474 cells, which are ER+/HER2+, and a trastuzumab-resistant derivative of these cells, BT474-HR20 (Figure 4). These HR20 cells have been chronically treated with 20ug/mL trastuzumab, and have developed resistance through a mechanism of HER3/IGF1R activation, such that HER2 is still expressed, and the downstream activation of HER2/HER3 target genes is also maintained. Figure 4 shows results of a test of combining enza with trastuzumab. Our collaborator Dr. Bolin Liu has determined that the trastuzumab resistant BT474 cells grow better *in vivo* than the parental cell line. All HER2+ cell lines in general are all known for not growing well *in vivo*. These cells have a higher take rate in NOD/SCID mice. We performed an *in vivo* study using the trastuzumab resistant BT474-HR20 cells. We set up the experiment with 4 treatment groups: Veh, Enza, Trastuzumab, Enza+Trastuzumab. Mice were randomized to treatment groups after tumors grew to a size of approximately 50mm³. At day 47 post-treatment, mice in the vehicle group and

the enza group were sacrificed. There was a statistically significant difference in tumor weight between these two groups, with the enza-treated tumors having lower tumor weight (Figure 5).

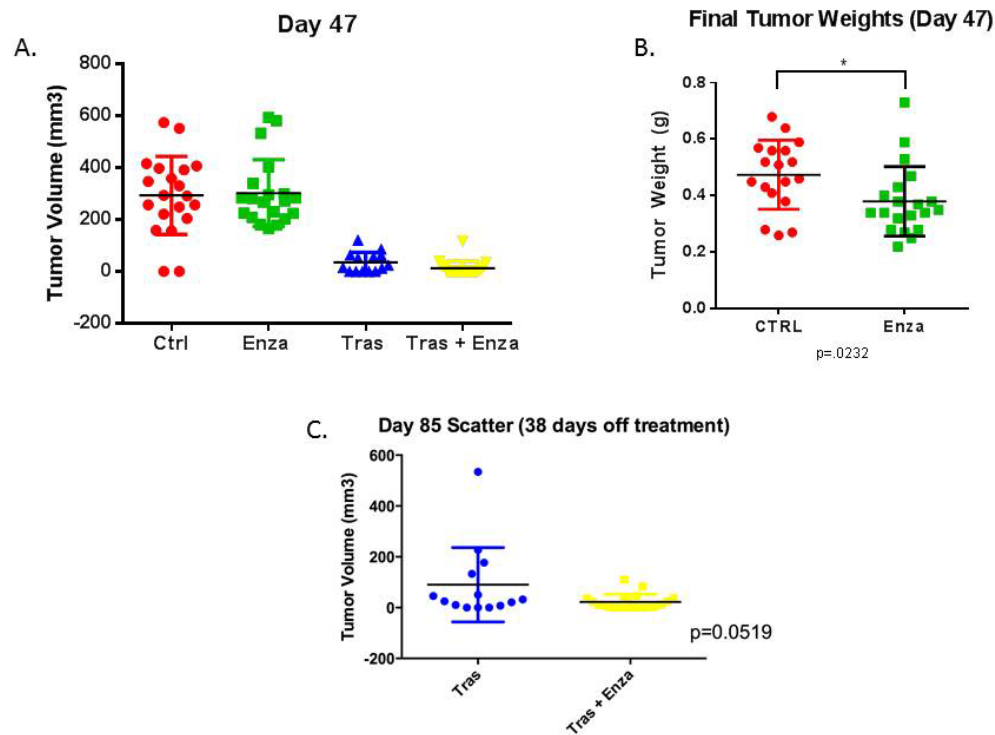
Figure 4. Enzalutamide shows synergy with trastuzumab in both trastuzumab responsive and resistant HER2+/ER+ BT474 breast cancer cells. BT474 cells (HER2+/ER+) and trastuzumab-resistant BT474-HR20 cells were treated alone or in combination with enzalutamide and trastuzumab. Cells were grown for 5 days and then proliferation was measured by crystal violet assay. 3D bar graphs depict percent inhibition at dose combinations indicated. Synergy was calculated with Calcsyn software. A Combination index <1 indicates synergy, 1 indicates an additive effect, and >1 indicates an antagonistic effect. The dose reduction index measures the factor by which a drug dose can be reduced when it is combined with another drug, in order to get the same effect as single agent.



At day 47, the mice that got vehicle (no drug treatment) and those that only had enzalutamide had to be sacrificed, but final tumor weight was significantly less in the enza treated group. Mice in the trastuzumab only and enza+trastuzumab groups were not killed since they had a significantly lower tumor burden, but they were taken off drug, and weekly caliper measurements were performed to determine if there would be a difference in tumor re-growth. While not yet statistically significant, there is a trend towards the enza+trastuzumab treated mice having slower regrowth of tumors (Figure 5). This would indicate that even though the tumor volume was not different at day 47 between the trastuzumab only and the trastuzumab plus enzalutamide groups, the combination with enza may be killing more cells and therefore will reduce the rate of recurrence, but this experiment is still ongoing. This work is very important because there is a trial for enza plus trastuzumab in HER2+ disease that is just starting in breast cancer. This might tell us that even if there is not an obvious decrease in tumor size (if this is done in the neoadjuvant setting) or reduction in metastatic tumor burden, there may be some benefit still if it lowers the chance of recurrence or rate of progression in HER2+, particularly those that are not very sensitive to trastuzumab. Now there are other options for HER2 positive disease, but they all do target HER2 or HER3 and they may all benefit from the combination with an anti-androgen because we see that AR upregulates HER2 and HER3 (as we have shown in previous progress reports). Our in vivo work

shown in Figure 5 that is still ongoing will be added to a paper in preparation on targeting AR in HER2+ breast cancer.

Figure 5. HER2+ BT474-HR20 xenografts respond to trastuzumab and enzalutamide, but the combination achieves a sustained response after treatment is ceased. (A) Mice were treated for 47 days, and tumor volume was measured 1x/week. Day 47 tumor volumes are shown. **(B)** All mice in the vehicle and Enza groups were sacrificed at day 47, and tumor weights were measured. There was a statistically significant difference in tumor weight between the vehicle group and the Enza group. **(C)** Mice in trastuzumab and trastuzumab+Enza groups were taken off treatment at Day 47, and tumors were allowed to regrow. Tumor volumes are being measured 1x/week, with day 85 shown (38 days after treatment cessation). The difference in tumor volume between the two groups is approaching statistical significance ($p=0.519$)



Task 4: Examine enzalutamide in combination with an mTOR inhibitor (Afinitor/everolimus) (90 % complete). Further mechanistic studies in this Task have revealed that there is cross-regulation between AR and the HER2/PI3K/Akt/mTOR signaling axis. In previous progress reports we showed *in vitro* that enzalutamide synergizes with everolimus in HER2+ and TNBC cells. Everolimus causes an upregulation in AR protein expression, as well as phospho-HER2 and phospho-HER3 (through AR); addition of enzalutamide plus everolimus abrogates these effects upregulation. We have demonstrated a synergistic interaction between the mTOR inhibitor everolimus and enzalutamide in several HER2+ breast cancer cell lines. We now show that there is a cell-line specific effect of everolimus on the expression of phospho-HER2, phospho-HER3, and AR, and that this effect is abrogated by enzalutamide (Figures 6 and 7). However this effect appears to be cell line-specific, and was not observed in the HER2+/ER- cell line SKBR3 (Figure 7).

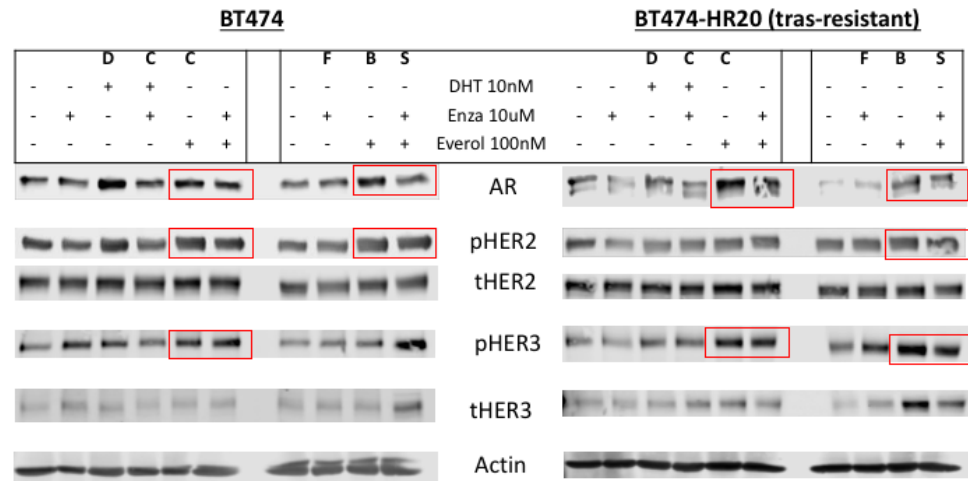
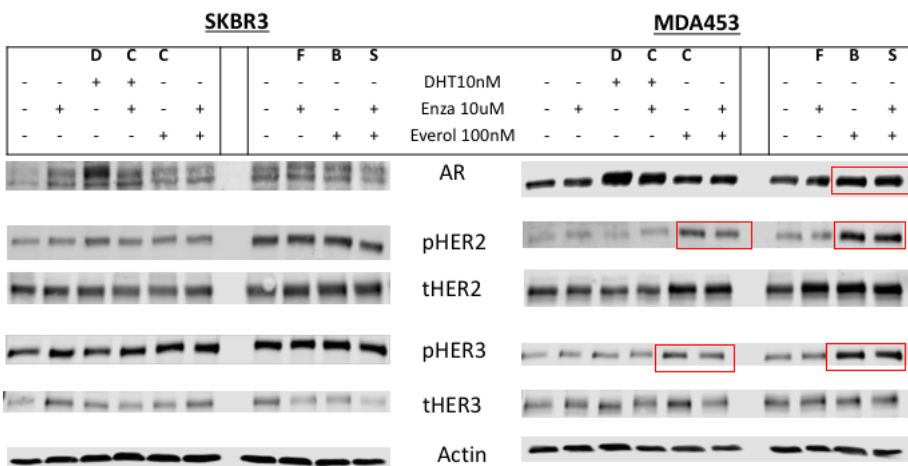


Figure 6. The mTOR inhibitor everolimus upregulates AR and pHER3 in BT474 cells and trastuzumab-resistant BT474-HR20 cells. Cells were either grown in charcoal stripped serum (DCC) or full serum (FBS) and treated with enzalutamide, everolimus, and DHT (only DCC-treated cells). Whole cell lysate was harvested at 48 hours,



and western Immunoblot was performed to detect total AR, pHER2, HER2, pHER3, HER3, and actin (loading control).

Figure 7. The mTOR inhibitor everolimus upregulates AR, pHER2, and pHER3 in ER-/HER2+ MDAMB453 cells, but not SKBR3 cells. Cells were either grown in charcoal stripped serum (DCC) or full serum (FBS) and treated with enzalutamide, everolimus, and DHT (only DCC-treated cells). Whole cell lysate was harvested at 48 hours, and western Immunoblot was performed to detect total AR, pHER2, HER2, pHER3, HER3,

and actin (loading control).

We would like to model whether enza and everolimus synergize in vivo as well. We are also seeing promising results with enza combined with palbocyclib the CDK4/6 inhibitor in vitro, which we obtained from Pfizer.

Task 5. In true TNBC cell lines and explants that retain AR, enzalutamide will be evaluated alone and in combination with chemotherapy and everolimus, in vitro and in vivo. (85% completed)

Doctoral candidate Valerie Barton, is currently writing up another manuscript on a unique role for AR in supporting a cancer stem-cell like population in TNBC. Valerie made the unique observation that AR messenger RNA and protein increased when TNBC cells were grown in forced suspension (Figure 8). She had already published that treatment with enza decreased growth on soft agar (Barton V et al 2015), but this new result begged the question of whether TNBC might be even more reliant on AR when the cells are metastasizing, which requires them to be resistant to death by detachment (in other words to be anchorage independent). AR transcriptional activity also increased in the cells grown in forced suspension for only 24 hours (Figure 9).

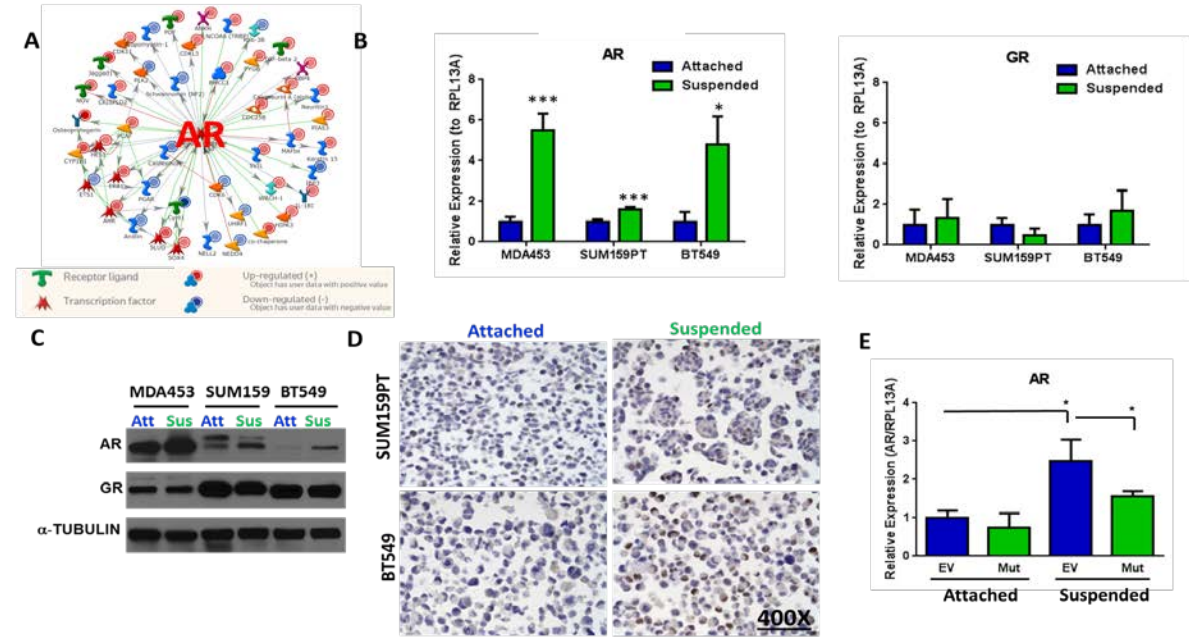
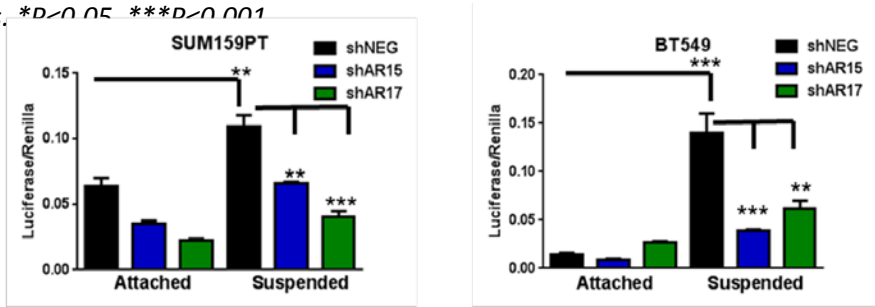


Figure 8– condition forced su: quantitat transduce for 24 hours. **Figure 9 – Androgen Receptor (AR) transcriptional activity increases in forced suspension compared to attached conditions.** TNBC cells were transduced with a non-targeting control (shNEG), or shRNAs targeting AR (shAR15, shAR17) and then transfected with a luciferase reporter containing an AR response element. The luciferase assay was performed following 24 hours in forced suspension. **P<0.01, ***P<0.001



Valerie then asked the question of whether manipulating AR would affect the ability of the cells to survive in suspension (Figure 10) or affect the population of cancer stem-like cells as detected by flow cytometry (FACs) analysis of aldehyde dehydrogensase activity or mammosphere formation (Figure 11). Since stem like cells are thought to be the cells capable of tumor initiation, we performed an *in vivo* assay for the frequency of tumor initiating cells to determine if enzalutamide treatment affects the ability of TNBC cells to initiate tumors. Indeed the frequency of tumor initiation (as measured by palpation or detection of labelled cells by IVIS imaging (Figure 12).

We also are interested in whether enzalutamide would be more effectively given simultaneously or subsequent to chemotherapy, since there is rationale for both approaches. Therefore, we started an experiment using the TNBC cell line SUM159 that is a true triple negative as opposed to a “LAR or luminal AR TNBC” that has a lot of AR that substitutes for ER to give a more luminal molecular profile. We have shown in a previous publication (Barton V et al Mol Can Therapeutics, 2015) that the SUM159s are sensitive to enza even though they only have low level AR protein expression. In Figure 13 we show an ongoing experiment in which we let the SUM159 xenograft tumors at the orthotopic site grow to an average size of 50 mm cubed then gave a 5 day treatment with either paclitaxel alone, together with enzalutamide simultaneously or paclitaxel followed by enzalutamide. Although the experiment is still ongoing we do see a significant difference in tumor cell viability as measured by IVIS imaging in the group that got the simultaneous treatment compared to paclitaxel only, while the subsequent enza is not yet different form the chemotherapy only group. We hope to perform similar experiments with the TNBC PDXs that we have aquired (see Figure 14).

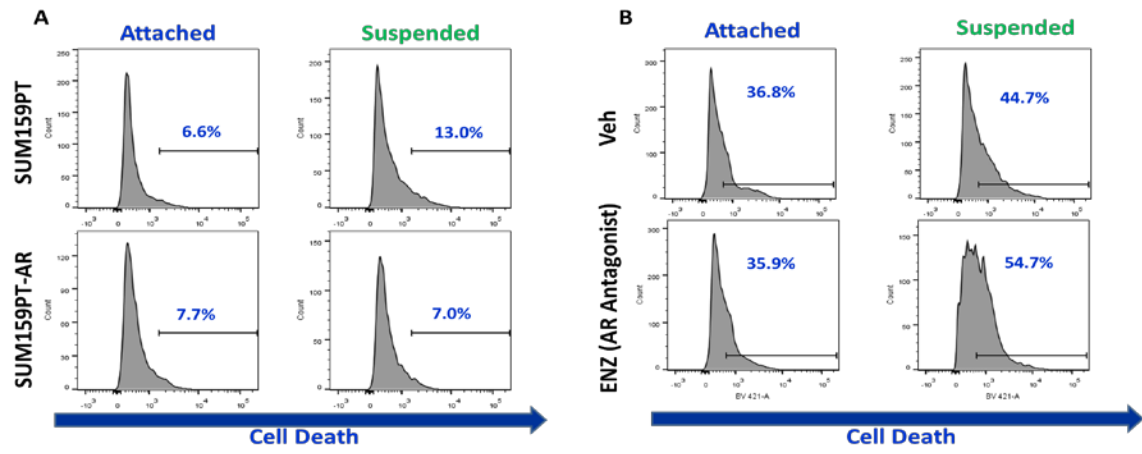


Figure 10 – Androgen Receptor (AR) overexpression increases and Enzalutamide (ENZ) decreases survival in suspension. Percentages indicate cells stained with Viability Factor (% dead). **(A)** SUM159PT cells overexpressing AR (SUM159PT-AR) compared to parental cells following a 3 day forced suspension. **(B)** SUM159PT cells treated with 20 mM ENZ or vehicle control (Veh) during a 3 day forced suspension.

Figure 11 – Androgen Receptor (AR) inhibition decreases and AR overexpression or treatment with DHT increases a cancer stem cell-like population. **(A)** FACS analysis of ALDH activity (+/- DEAB) following 5 days in attached versus suspended conditions. **(B)** FACS analysis of ALDH activity following AR knockdown. **(C)** Mammosphere formation efficiency (MFE) following AR overexpression or knockdown. **(D)** MFE and FACS analysis of ALDH activity following a 5d treatment with Veh, 20 mM Enza and/or 10 nM DHT.

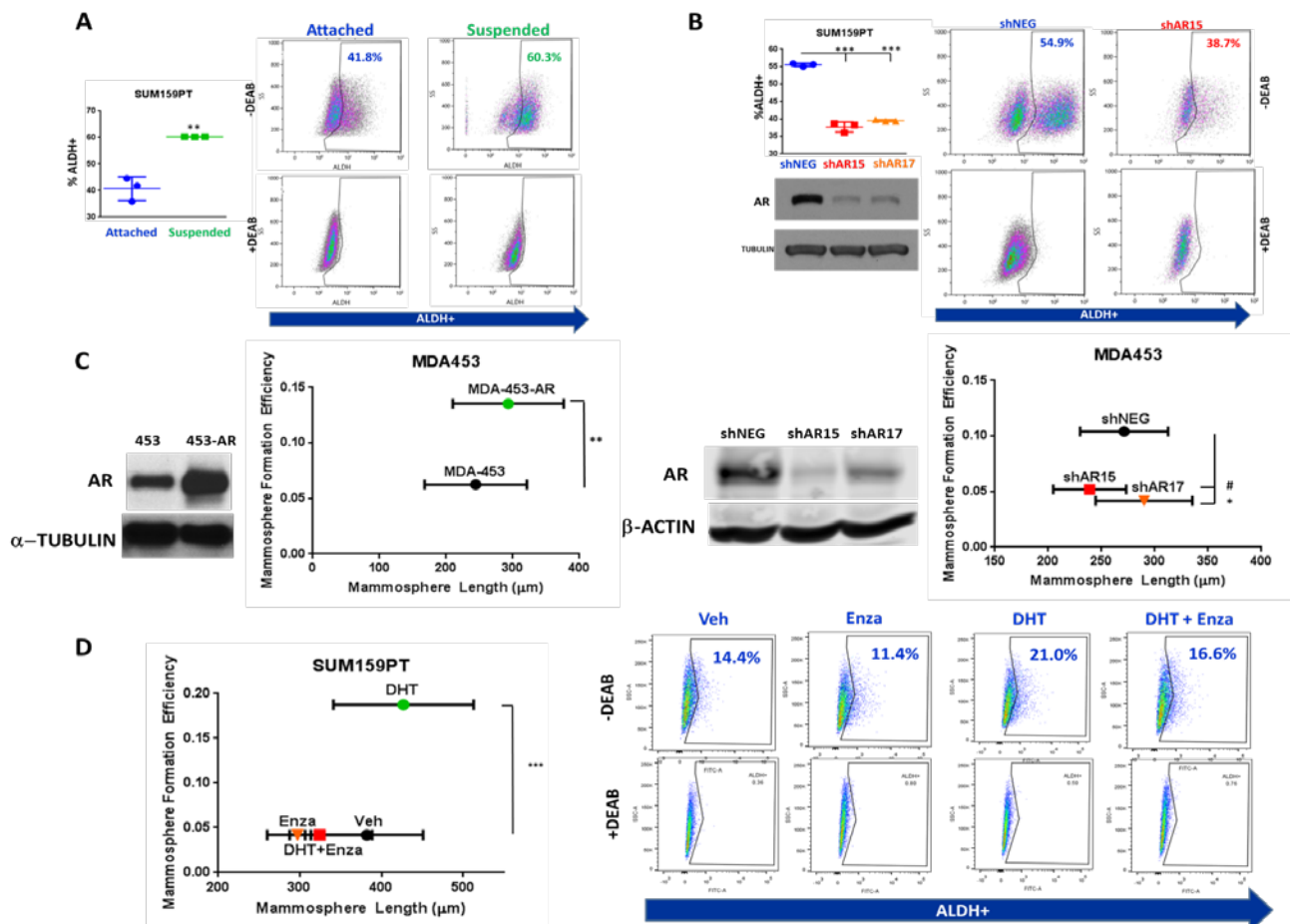


Figure 12– Treatment with Enzalutamide (Enza) decreases tumor initiation frequency. **(A)** Luciferase-tagged SUM159PT cells were pre-treated with vehicle control (Veh) or 20 mM Enza for three days prior to injections. **(B)** Table displaying tumor formation at each dilution and stem cell frequencies. **(C)** Luminescent overlay of tumor formation at day 14.

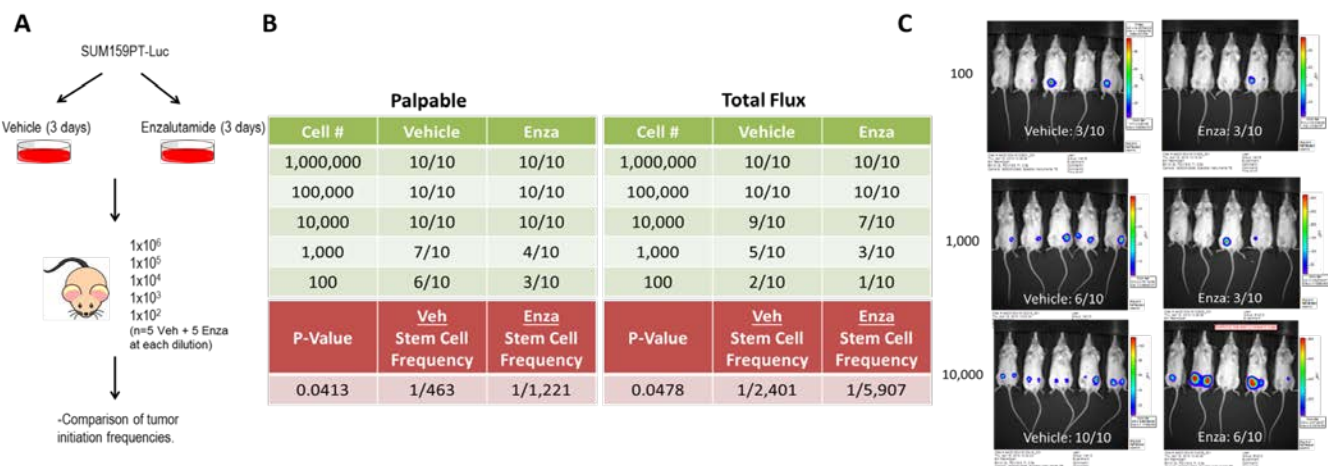
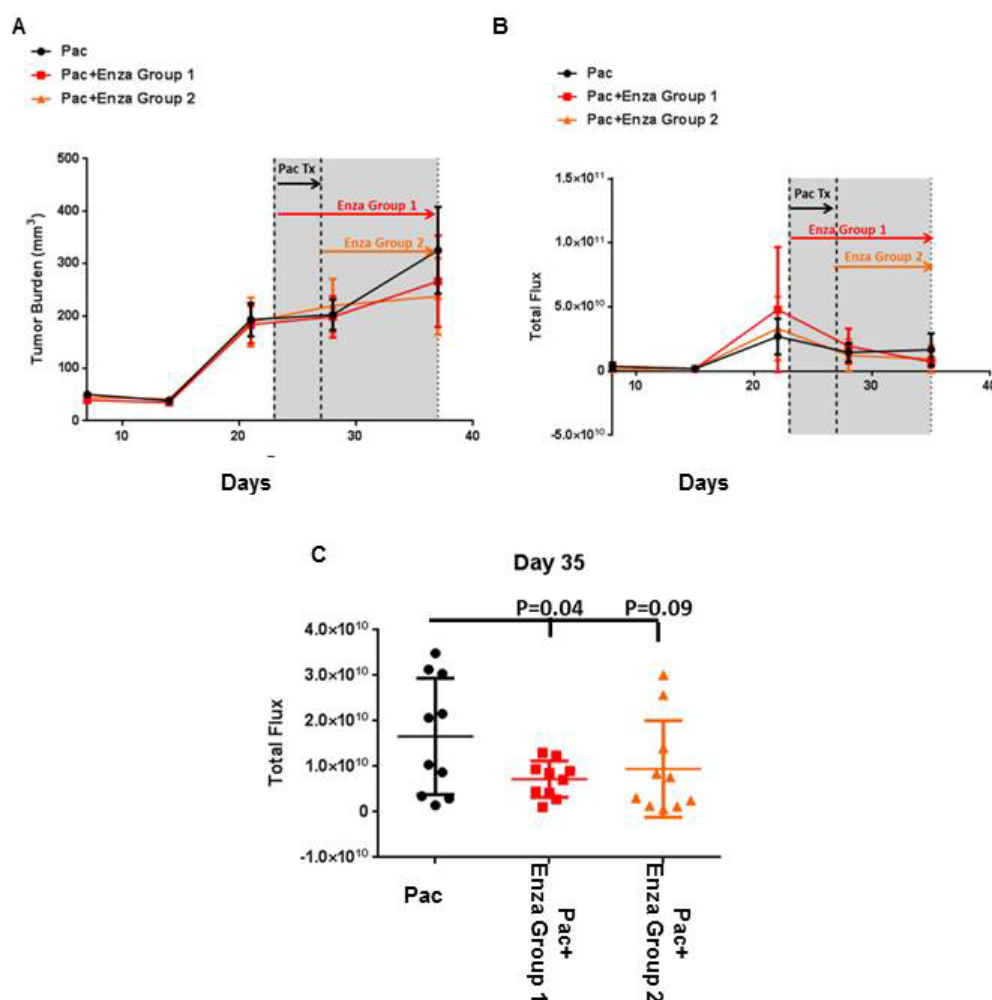


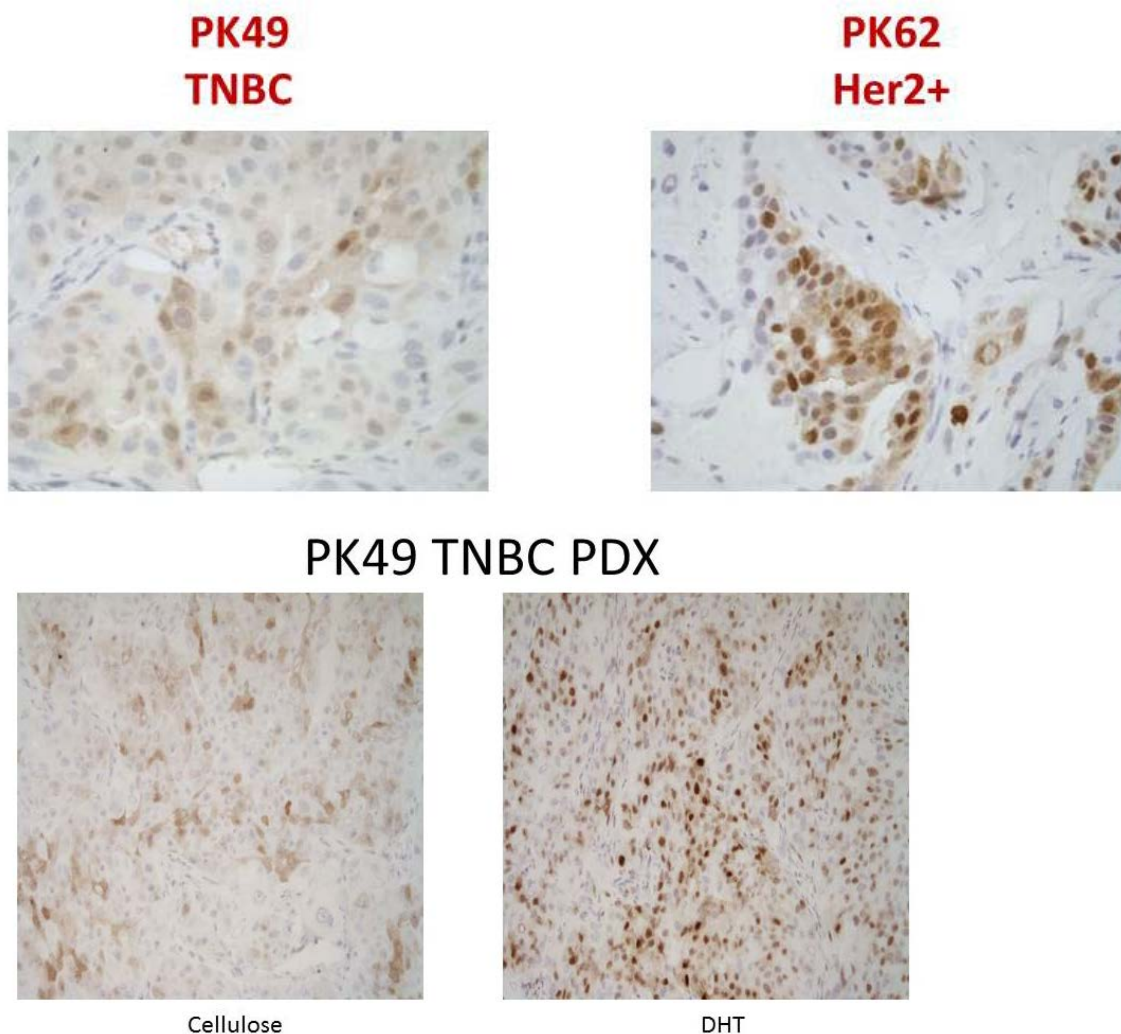
Figure 13. Ongoing study of SUM159PT xenografts treated with paclitaxel and enzalutamide. Luciferase expressing SUM159PT cells were bilaterally injected (day 1) into the mammary fat pads of nude mice. Following randomization (day 23) mice were treated for 5 days with paclitaxel (Pac, n=10), Pac and enzalutamide (Pac+Enza group 1, n=10), or Pac followed by Enza (Pac+Enza group 2). Total tumor burden by caliper measurements (A) or luciferase activity (B). C) T-test comparing total flux of Pac and Pac+Enza Group 1 or Pac+Enza Group 2 at study day 35.



Lastly, we have been performing immunohistochemistry for AR on various HER2+ and TNBC patient derived xenografts (PDX). Figure 12 top shows AR protein staining in TNBC and HER2+ PDXs from our collaborator Dr. Carol Sartorius here at the University of Colorado. We discovered that the TNBC AR expression gradually decreased over two passages in mice. We therefore put the TNBC PDX PK49 into 2 NOD/SCIDgamma mice with cellulose pellets and two with DHT pellets and then upon tumor growth we resected the tumors and froze half and formalin fixed paraffin embedded the other half. Upon staining for AR, we observed a significant increase in nuclear AR positivity and intensity of expression (Figure 12

bottom). We also saw this phenomenon in a TNBC PDX from Baylor (not shown). Now we plan to determine if these PDX will respond to enza more strongly if they are pretreated with DHT. This could be very clinically relevant if the clinical benefit from enza in TNBC patients reported by Medivation and Astellas and Dr. Tiffany Traina at Memorial Sloan Kettering at ASCO 2015 could be improved by pretreating the patients with a dose of androgen prior to enzalutamide. We will also perform an in vivo experiment with the HER2+ PK62 PDX to determine if enzalutamide and trastuzumab synergize to inhibit tumor growth in this model as we showed above in HER2+ cell lines.

Figure 14. Positive AR staining in a TNBC PDX and a HER2+ PDX. *Top:* Immunohistochemistry for AR was performed with the Ventana anti-AR antibody on FFPE sections of two PDX that have been passaged in mice twice. The TNBC showed some cytoplasmic staining and very low percent cells positive for nuclear AR, while the HER2+ PDX has almost 100% 2+ staining. **Bottom:** Because the original specimen from the patient had higher AR levels in the TNBC PK49 tumor, as did the first passage in mice, whereas this second passage was lower, and in order to determine if AR was functional, we implanted two mice with pellets of 8mg DHT and 2 with equal amount of cellulose.



Preclinical Aim 2. Using samples collected from the xenograft studies, examine if and how the mechanism of action by which enzalutamide works in the various subtypes of breast cancer.

- **Task 1.** Perform IHC on xenograft tumors for AR, ER, Her3, BrdU, FOXA1, SDF1, Cyr61. Months 12-18. We continue to perform IHC for all of these protein from the xenograft experiments on ER+, HER2+ and TNBC where relevant. We are still analyzing results of staining of the ER regulated proteins SDF1 and Cyr61, which we

do see to be affected by AR inhibition as reported in previous progress reports, but the antibodies for IHC are not optimal.

- **Task 2.** Make RNA from xenografts. Perform profiling. Analyze data. Months 15-18 (60% completed)

We continue to analyze RNA profiling from ER+ and TNBC cell line xenografts. We want to compare our results to the ER+PDX (the PT12 grown with either E2 or DHT then with or without enzalutamide. We want to compare the SUM159 TNBC xenograft profiling we have done to the TNBC PDX that we show in figure 14 from Univ of Colorado and the one from Baylor. We have not yet finished the experiment with the BT474 HER+ line in vivo or the HER2+ PDX shown in figure 14, so we do not yet have RNA from those tumors. We hope to publish another paper on the genes regulated by AR in the various subtypes of breast cancer and how those are affected by enzalutamide treatment. This will also help us with preclinical Aim 3 below.

Preclinical Aim 3. Identify mechanisms of resistance to enzalutamide in triple negative breast cancers to elucidate pathways that impinge on the AR pathway to potentially target in combination with enzalutamide.

- **Task 1.** Sequence 3 AR+ triple negative cell lines resistant and 3 that are sensitive. Months 18-24 (25% completed).

Since the TNBC cell lines that we have studied so far are sensitive to enzalutamide and we have found it to particularly affect growth on soft agar (Barton V et al 2015), we have not performed sequencing of all of these yet because we are still trying to figure out the best conditions and timing. We are also exploring another approach, which is to chronically treat the cells with enza to generate resistant lines. We have taken this approach with the MDA-MB-453 TNBC line which we showed in Cochrane et al 2014 to be very responsive to enza in vitro and in vivo. We now have a resistant line and we will sequence it and compare to the parental line. We did do mutational analysis on the resistant line and it does not have the F876L mutation that has been reported to confer resistance to enzalutamide in prostate cancer cells and patient tumors. So far this mutation has not been found in breast cancers; however we are still analyzing the post-treatment biopsies that we obtained from the enzalutamide clinical trial described in Dr. Elias's report. It is likely that the cell line that we have rendered resistant is resistant via a different mechanism other than this AR mutation, but we have not sequenced the whole gene yet. It could also now be dependent on a completely different pathway other than AR and only RNA-seq would potentially tell us that, so we will proceed to do that compared to the wild-type line and continue to generate additional TNBC resistant lines.

References:

Dawn R. Cochrane, Sebastian Bernales, Britta M. Jacobsen, Diana M. Cittelly, Erin N. Howe, Nicholas C. D'Amato, Nicole S. Spoelstra, Annie Jean, Paul Jedlicka, Kathleen C. Torkko, Andy Protter, Anthony D. Elias and **J. K. Richer**. Role of the Androgen Receptor in Breast Cancer and Preclinical Analysis of Enzalutamide. *BREAST CANCER RESEARCH* 2014 Jan 22;16(1). PMID: 24451109 Designated as Highly Cited by the journal Breast Cancer Research.

Barton VN, Gordon MA, Christenson J, D'Amato N, Richer JK. Androgen receptor biology in triple negative breast cancer: A case for AR+ and quadruple negative disease subtypes. *Hormones and Cancer*, 2015 July 23. PMID: 26201402

BartonVN, D'Amato NC, Gordon MA, Lind HT, Spoelstra NS, Babbs B, Heinz RE, Elias A, Jedlicka P, Jacobsen BM, Richer JK. Multiple molecular subtypes of triple negative breast cancer critically rely on androgen receptor and respond to Enzalutamide in vivo. *Mol Cancer Ther*.015 Mar;14(3):769-78. Epub 2015 Feb 23. PMID: 25713333

What opportunities for training and professional development has the project provided?

Cancer Biology Graduate Program doctoral candidate Valerie Barton and postdoctoral fellows Nicholas D'Amato and Michael Gordon have presented the following oral and poster presentations on this project at various national and local meetings:

Nicholas D'Amato Poster presentations:

D'Amato, NC, D Cochrane, N Spoelstra, A Chitrakar, B Babbs, A Protter, A Elias, and J Richer. (Mar 2014) Inhibiting Androgen Receptor Nuclear Localization Decreases ER Activity and Tumor Growth in ER+ Breast Cancer. University of Colorado Postdoctoral Research Day, Aurora, CO. * won best overall poster award.

D'Amato, NC, B Jacobsen, N Spoelstra, B Babbs, A Elias, J Gertz, and J Richer. (Oct 2014) Inhibiting Androgen Receptor Nuclear Localization Decreases Estrogen Receptor Activity and Tumor Growth in ER+ Breast Cancer. Cancer Biology Training Consortium Retreat, Estes Park, CO.

D'Amato, NC, B Jacobsen, N Spoelstra, B Babbs, A Elias, J Gertz, and J Richer. (Dec 2014) Inhibiting Androgen Receptor Nuclear Localization Decreases Estrogen Receptor Activity and Tumor Growth in ER+ Breast Cancer. San Antonio Breast Cancer Symposium, San Antonio, TX.

D'Amato, NC, B Jacobsen, N Spoelstra, B Babbs, A Elias, J Gertz, and J Richer. (Aug 2015) Inhibiting Androgen Receptor Nuclear Localization Decreases Estrogen Receptor Activity and Tumor Growth in ER+ Breast Cancer. Hormone-Driven Cancers Gordon Research Conference, Newry, ME.

Nicholas D'Amato Oral presentations:

Invited oral symposium presentation: Targeting Androgen Receptor in Her2-Driven Breast Cancer. Endocrine Society Annual Meeting, June 2013, San Francisco, CA.

Invited short talk: Targeting Androgen Receptor to Inhibit ER+ Breast Cancer Growth. Keystone Nuclear Receptors Meeting, January 2014, Taos, NM.

The Role of Androgen Receptor in Estrogen Receptor Activity in ER+ Breast Cancer. Functional Development of the Mammary Gland Program Project Grant Retreat, February 2014, Aurora, CO.

Targeting Androgen Receptor to Inhibit ER+ Breast Cancer Growth. UC Denver Anschutz Medical Campus Hormone Related Malignancies Retreat, March 2014, Aurora, CO.

Inhibiting Androgen Receptor Nuclear Localization Decreases ER Activity and Tumor Growth in ER+ Breast Cancer. UC Anschutz Medical Campus Postdoctoral Research Day, March 2015, Aurora, CO.

Inhibiting Androgen Receptor Nuclear Localization Decreases ER Activity and Tumor Growth in ER+ Breast Cancer. Division of Endocrinology Research Conference, March 2015, Aurora, CO.

Invited oral symposium presentation: Inhibiting Androgen Receptor Nuclear Localization Decreases Estrogen Receptor Activity and Tumor Growth in ER+ Breast Cancer. Hormone-Driven Cancers Gordon Research Conference, August 2015, Newry, ME.

Valerie Barton Oral presentations:

Barton VN, D'Amato N, Richer JK. Androgen receptor (AR) supports a cancer stem cell-like population in AR+ triple negative breast cancer. Oral presentation at Gordon Research Conference, Hormone-Dependent

Cancers, Newry ME, August 2015.

Barton VN, D'Amato N, Richer JK. Androgen receptor in triple negative breast cancer. Oral presentation at Obesity-Cancer Retreat, University of Colorado Anschutz Medical Campus, August 2015.

Barton VN, D'Amato N, Richer JK. Androgen receptor (AR) supports expansion of cancer stem-like cells in AR+ triple negative breast cancer. Oral presentation at Hormone Related Malignancies Symposium, University of Colorado Anschutz Medical Campus, May 2015.

Barton VN, D'Amato N, Richer JK. Endocrine therapy for triple negative breast cancer. Oral presentation at Pathology Grand Rounds, University of Colorado Anschutz Medical Campus, October 2014.

Valerie Barton Poster presentations:

Barton VN, D'Amato NC, Gordon MA, Jacobsen BM, Richer JK. Multiple subtypes of triple negative breast cancer are dependent on androgen receptor. Presented at San Antonio Breast Cancer Symposium, December 2014.

Barton VN, D'Amato NC, Richer JK. Androgen receptor (AR) supports a tumor initiating population in AR+ triple negative breast cancer. Presented at Gordon Research Conference on Hormone Dependent Cancers, August 2015.

Michael Gordon Poster presentations:

Gordon MA, D'Amato N, Gu H, Liu B, Elias A, Richer JK. Targeting multiple pathways in breast cancer: Androgen Receptor, HER2, and mTOR. Presented at San Antonio Breast Cancer Symposium, December 2014.

Gordon MA, D'Amato N, Gu H, Liu B, Elias A, Richer JK. The anti-androgen enzalutamide synergizes with trastuzumab and everolimus to inhibit breast cancer growth via distinct mechanisms. Selected for oral poster preview presentation, Endocrine Society Annual Meeting, March 2015.

▪ **How were the results disseminated to communities of interest?**

June 2013 Nicholas D'Amato gave a presentation to a group of donors for the **Colorado Springs, Colorado Chapter of the American Cancer Society** regarding the AR in breast cancer project.

July 2014 Nicholas D'Amato was invited to **Anchorage, AK** as the keynote speaker for an event for a new local chapter of the **American Cancer Society** - Making Strides kickoff event. I presented my work in lay terms to an audience of 150+ people, and also had separate meetings with physicians, caregivers, and local ACS staff to discuss

Dr Richer gave the following lectures:

May 2014	MD Anderson Breast Cancer Research Program Retreat One of two Keynote Speakers with Thea Tilsty "Targeting Androgen Receptors in a Subset of Triple Negative Breast Cancers."
May 2015	Bayer Scientific Advisory Board Whippany, NJ "Landscape of Androgen Receptors in Breast Cancer Subtypes"
July 2015	Virginia Commonwealth University Department of Pathology Grand Rounds
Aut 2015	Medivation Inc. SF, CA Update on Androgen Receptors in Breast Cancer Preclinical Models

▪ **What do you plan to do during the next reporting period to accomplish the goals?**

As shown in this report we have completed some studies with patient derived xenografts (PDX) at the orthotopic site. We are also testing how enzalutamide affects metastases of ER+ and TNBC and that experiment is still ongoing. For TNBC we are also testing to see if enza would provide the best benefit if it is given at the same time as chemotherapy or if it should be given subsequent to chemotherapy. There is a very clinically relevant issue since the clinical trial of enzalutamide in TNBC that was company sponsored, but run out of Memorial Sloan Kettering by Tiffany Traina did show clinical benefit in metastatic disease that had recurred on chemotherapy. Likely the next trial will be to test first line therapy with either chemotherapy alone or chemo plus enzalutamide, so any preclinical data regarding the timing (whether to give the two simultaneously or give enzalutamide subsequent to chemo) will be very helpful to clinical trial design. Furthermore in this progress report we show data from two TNBC PDX that had very low AR expression that increases substantially when we give androgen (DHT) to the mice. We are now performing an experiment to determine if these two PDX will respond better to enzalutamide if they have a pretreatment with DHT. This could definitely translate to the clinic if one does of an androgen would improve response to the antiandrogen in women with AR+ TNBC. This experiment is ongoing. We would also like to determine if enzalutamide affects metastasis of these TNBC PDX if we disassociate the cells and let them disseminate via intracardiac injection.

The last ongoing experiment is with the HER2+ BT474 cells. We have finally got a version of these cells that was selected *in vivo* to be trastuzumab resistant to grow well in NOD/SCID mice. Although they are resistant to trastuzumab *in vitro*, they are responding to trastuzumab *in vivo*, but respond to the combination of trastuzumab and enzalutamide better. We would like to do molecular profiling of the tumors to identify the mechanism behind this effect. We would also like to treat the mice with estrogen since this line is ER+ and HER2+ and also has high AR protein expression. They tumors will likely grow even better in the presence of estrogen and we can determine if the enzalutamide is even more effective when opposing estrogen-stimulated proliferation, as we have seen it to be in the ER+/AR+ xenografts and ER+/AR+ PDX models.

In the ER+/AR+ cell line or xenograft models, we would like to test the combination of fulvestrant with enzalutamide *in vivo* since we observe synergy *in vitro* and that will most likely be the next next clinical trial in ER+ disease. We did put the tamoxifen resistant MCF7 cells *in vivo* as we said in last year's annual review and that experiment was very promising. Since Fulvestrant is the standard of care after recurrence on tamoxifen and aromatase inhibitors, it makes sense to try this combination *in vivo*.

Last year in this report we said we would perform the to determine if keeping AR from the nucleus with Enz will change where ER binds to chromatin and we got very convincing results from that experiment that showed two things: one that the ER binding to DNA is diminished when AR is excluded from the nucleus using enzalutamide or MJC13 and secondly that AR binds to DNA almost as well with estrogen as it does with androgens and that some unique sites are bound in the presence of estrogen. This is in the paper that we are almost ready to send that is in the appendix (Figures 2 and 3). However, in anticipation of a reviewer comment, we would like to also do these experiments in another ER+/AR+ breast cancer cell line that is commonly used by other laboratories, the ZR75.

IMPACT:

▪ **What was the impact on the development of the principal discipline(s) of the project?**

These studies are helping to determine the role of androgen receptors in breast cancer and whether new anti-androgens might be utilized as therapy for breast cancers that fail to respond or reoccur while women are on current therapies such as anti-estrogens, trastuzumab or chemotherapy. These studies have provided preclinical evidence that the anti-androgen enzalutamide could serve as the first effective targeted therapy for a subset of triple negative breast cancers (TNBC). TNBC is the most aggressive type of breast cancer and there is currently no effective treatment for TNBCs with de novo or acquired resistance to chemotherapy. Our ongoing studies regarding timing (concurrent treatment with chemotherapy and enzalutamide versus sequential) will provide valuable information for upcoming clinical trials. Our studies on the effect of enzalutamide on the process of metastasis will also inform clinical trial design as will the studies of combined therapy with fulvestrant in breast cancer patients with ER+ disease.

- **What was the impact on other disciplines?** Our studies of how steroid hormone receptors affect each other is definitely pertinent to other cancers and development. Already other investigators are looking at the idea of examining the AR to ER ratio in lung cancer as well in conjunction with Medivation.
- **What was the impact on technology transfer?**
 - Transfer of results to entities in government or industry: The results of this project are also reported to our clinical industry partners Medivation Inc and Astellas Pharma who are running the clinical trials of enzalutamide in prostate and breast cancer. They are very interested in our preclinical results combining enzalutamide with other therapeutics currently being utilized in breast cancer since these results will guide the design of further industry or investigator initiated clinical trials. We filed a patent on the idea of looking at the AR to ER ratio in breast cancer and the company Ventana is close to signing an agreement to pay the filing fees in Europe and to contract some additional sponsored research to design a clinical test to examine the ration of these two receptors using their antibodies potentially simultaneously on the same section of tumor.
- **What was the impact on society beyond science and technology?**
- Since we have given reports of our research to several lay audiences in various community settings, we believe we are improving public knowledge regarding how hormones typically thought of as male hormones (such as androgens) are made by women and do affect women's health.

CHANGES/PROBLEMS: Nothing to Report

Changes in approach and reasons for change

- Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.
- **Actual or anticipated problems or delays and actions or plans to resolve them.** We found that AR protein expression in TNBC PDX models was decreasing even after two passages. However, we have now found that with androgen treatment, AR is re-expressed and we will now see if that confers an even better response to enzalutamide.
- **Changes that had a significant impact on expenditures.** Nothing to report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents** No significant changes, but we had to do a three year re-write of the protocol and that was approved

by the local IACUC and the DOD. We will send a copy of that approval to the DOD.

Significant changes in use or care of human subjects. None

- **Significant changes in use or care of vertebrate animals. None**
- **Significant changes in use of biohazards and/or select agents. None**

PRODUCTS:

Journal publications.

In addition to the Cochrane *et al* 2014 paper below, which was reported in the first Annual Progress report, we have published two manuscripts on AR in TNBC. One of these is a primary paper and one an invited review.

Dawn R. Cochrane, Sebastian Bernales, Britta M. Jacobsen, Diana M. Cittelly, Erin N. Howe, Nicholas C. D'Amato, Nicole S. Spoelstra, Annie Jean, Paul Jedlicka, Kathleen C. Torkko, Andy Protter, Anthony D. Elias and **J. K. Richer**. Role of the Androgen Receptor in Breast Cancer and Preclinical Analysis of Enzalutamide. *BREAST CANCER RESEARCH* 2014 Jan 22;16(1). PMID: 24451109 Designated as Highly Cited by the journal Breast Cancer Research.

Barton VN, Gordon MA, Christenson J, D'Amato N, Richer JK. Androgen receptor biology in triple negative breast cancer: A case for AR+ and quadruple negative disease subtypes. *Hormones and Cancer*, 2015 July 23. PMID: 26201402

BartonVN, D'Amato NC, Gordon MA, Lind HT, Spoelstra NS, Babbs B, Heinz RE, Elias A, Jedlicka P, Jacobsen BM, Richer JK. Multiple molecular subtypes of triple negative breast cancer critically rely on androgen receptor and respond to Enzalutamide in vivo. *Mol Cancer Ther.* 015 Mar;14(3):769-78. Epub 2015 Feb 23. PMID: 25713333

We have another paper that is almost ready to submit and it is attached in the appendix.

- **Books or other non-periodical, one-time publications.** Nothing to report.
- **Other publications, conference papers, and presentations.**

Dr. Richer presented the following seminars/lectures/posters:

Dr Richer gave the following lectures:

May 2014 **MD Anderson Breast Cancer Research Program Retreat** One of two Keynote Speakers with Thea Tilsty "Targeting Androgen Receptors in a Subset of Triple Negative Breast Cancers."

May 2015 **Bayer Scientific Advisory Board** Whippany, NJ "Landscape of Androgen Receptors in Breast Cancer Subtypes"

Aug 2015 **Medivation Inc.** SF, CA Update on Androgen Receptors in Breast Cancer Preclinical Model

Sept 2015 **The US Oncology Network McKesson Annual Science Forum**, Dallas, TX "Role of Androgen Receptors in Breast Cancer"

- **Website(s) or other Internet site(s):**

Expert Opinion piece in Oncology PracticeUpdate <http://www.practiceupdate.com/journalscan/9370> or http://prac.co/j/5960d32c-988b-423e-ba24-14ca5c8cc39a?elsca1=soc_share-this acknowledgement of federal support –no

Highlight of Cochrane DR et al **Breast Cancer Research** 2014 in Feb issue of 2014 NATURE REVIEWS CLIIICAL ONCOLOGY. acknowledgement of federal support –yes

- **Technologies or techniques.** None

- **Inventions, patent applications, and/or licenses**

Richer *et. al.*, PCT Patent Application WO 2014/031164 filed March 15, 2013, “Methods for Determining Breast Cancer Treatment.”

Protter and JK Richer, PCT Patent Application PCT/US2012/48471 Serial No. 14/236,036 filed on January 29, 2014 “Treatment of Breast Cancer.”

- **Other Products**

data or databases- we now have databases of genes expression data from the following experiments.

ER+ MCF7 breast cancer cells treated in vitro with vehicle, enzalutamide alone, estradiol alone (E2), E2 plus enzalutamide for 48 hrs.

ER+ MCF7 breast cancer cells grown as xenografts in nude mice treated with E2, E2 plus tamoxifen, or E2 plus enzalutamide.

HCC1806 TNBC breast cancer line treated in vitro with either vehicle, DHT, enzalutamide alone, DHT plus enzalutamide.

SUM159 treated in vivo.

Are working on two TNBC PDX treated in vivo with or without DHT

- **biospecimen collections;**

formalin fixed paraffin embedded xenograft tumors from the following experiments:

MCF7 tumors grown in nude mice and treated with either E2, E2 plus tamoxifen, E2 plus enzalutamide or in a separate experment, the same treatments plus the combination of E2 plus enzalutamide and tamoxifen.

Triple negative breast cancer (TNBC) cell line SUM159PT grown as xenograft tumors in mice treated with control rodent chow or enzalutamide containing chow.

TNBC cell line HCC1806 grown as xenograft tumors in mice treated with control rodent chow or enzalutamide containing chow.

- research material (e.g., Germplasm; cell lines, DNA probes, animal models); We have generated luciferase labelled breast cancer cell lines to image by IVIS and put nuclear red and green expression vectors in these lines to utilize the Incucyte machine to count the number of red or green nuclei to do real time proliferation assays with enzalutamide alone or in combination with standard therapies for breast cancer.
- **What individuals have worked on the project?**

Personnel	Role	Percent Effort	Nearest Person Month Worked	Contribution to Project	Funding Support: DOD Contract W81XWH-13-0090
Jennifer Richer, PhD	Partner Principal Investigator	40%	5	Oversees all experiments – helps with animal experiments, writes reports and edits manuscripts	X
Britta Jacobsen, PhD	Collaborator	50%	6	Provides daily oversight and helps with in vitro and in vivo experiments and; helped with animal protocol 3 year rewrite	X
Carol Sartorius, PhD	Collaborator	3%	.4	Provided PT14 PDX	X
Tzu Phang, PhD	Collaborator	5%	.6	Provides bioinformatics	X
Ann Thor, MD	Collaborator	2%	.2	pathologist	X
Susan Edgerton	Instructor	2%	.2	Pulls pathology samples, analyzes results of IHC	X
Haihua Gu, PhD	Collaborator	50%	4	Helps to oversee experiments, especially for Her2+ disease and everolimus signaling experiments	X
Nicole Spoelstra	Technician	66%	8	Performs IHC on FFPE samples	X

Valerie Barton	Graduate Student	100%	12	Directing TNBC experiments	X
Michael Gordon, PhD	PostDoc	100%	12	Performing everolimus studies	X
Beatrice Babbs	Animal Technician	100%	12	Ms. Babbs has provided mouse related care, caliper measuring xenograft tumors in mice and IVIS imaging.*	X
Ann Jean	Technician	50%	4	Ms. Jean provided support for the mouse experiments until she left the department in December, 2013*	X

Has there been a change in the active other support of the PD/PI (s) or senior/key personnel since the last reporting period? Nothing to Report."

No changes in active support for the PD/PI(s) or senior/key personnel.

- **What other organizations were involved as partners?** Medivation Inc. and Astellas Pharma are the Industry partners.

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Partnering PI, Dr. Anthony Elias has sent a separate report on the clinical progress.
- **APPENDICES:**

The attached appendix contains publications and abstracts referred to above

Multiple Molecular Subtypes of Triple-Negative Breast Cancer Critically Rely on Androgen Receptor and Respond to Enzalutamide *In Vivo*

Valerie N. Barton¹, Nicholas C. D'Amato¹, Michael A. Gordon¹, Hanne T. Lind¹, Nicole S. Spoelstra¹, Beatrice L. Babbs¹, Richard E. Heinz¹, Anthony Elias², Paul Jedlicka¹, Britta M. Jacobsen¹, and Jennifer K. Richer¹

Abstract

Triple-negative breast cancer (TNBC) has the lowest 5-year survival rate of invasive breast carcinomas, and currently there are no approved targeted therapies for this aggressive form of the disease. The androgen receptor (AR) is expressed in up to one third of TNBC and we find that all AR⁺ TNBC primary tumors tested display nuclear localization of AR, indicative of transcriptionally active receptors. While AR is most abundant in the "luminal AR (LAR)" molecular subtype of TNBC, here, for the first time, we use both the new-generation anti-androgen enzalutamide and AR knockdown to demonstrate that the other non-LAR molecular subtypes of TNBC are critically dependent on AR protein. Indeed, AR inhibition significantly reduces baseline proliferation, anchorage-independent growth, migration, and invasion and increases apoptosis in four TNBC lines (SUM159PT, HCC1806, BT549, and

MDA-MB-231), representing three non-LAR TNBC molecular subtypes (mesenchymal-like, mesenchymal stem-like, and basal-like 2). *In vivo*, enzalutamide significantly decreases viability of SUM159PT and HCC1806 xenografts. Furthermore, mechanistic analysis reveals that AR activation upregulates secretion of the EGFR ligand amphiregulin (AREG), an effect abrogated by enzalutamide *in vitro* and *in vivo*. Exogenous AREG partially rescues the effects of AR knockdown on proliferation, migration, and invasion, demonstrating that upregulation of AREG is one mechanism by which AR influences tumorigenicity. Together, our findings indicate that non-LAR subtypes of TNBC are AR dependent and, moreover, that enzalutamide is a promising targeted therapy for multiple molecular subtypes of AR⁺ TNBC. *Mol Cancer Ther*; 14(3): 769–78. ©2015 AACR.

Introduction

Triple-negative breast cancer (TNBC) constitutes 10% to 20% of invasive breast carcinomas and has the lowest 5-year survival rate compared with other breast cancer subtypes (1). 12% to 28% of patients with TNBC achieve a pathological complete response following neoadjuvant chemotherapy and have a good prognosis (2, 3). However, patients with TNBC and residual disease have a significantly worse overall survival than patients with non-TNBC subtypes and residual disease (2). The discrepancy in survival between patients with TNBC and non-TNBC with residual disease is exacerbated by the absence of effective targeted therapy for TNBC. TNBC lacks estrogen receptor (ER) and progesterone receptor (PR) expression as well as HER2 amplification and thus is unresponsive to traditional endocrine- or HER2-directed therapies that improve overall survival in other breast cancer subtypes.

Although TNBC lacks the hormone receptors traditionally associated with breast cancer, many TNBCs express other hormone receptors, including the glucocorticoid receptor (4) and androgen receptor (AR). AR, a ligand-activated nuclear hormone transcription factor (5), is expressed in 12% to 36% of TNBC (6–9).

A defining role for AR and AR-regulated genes in the molecular biology and classification of breast cancer was established by microarray profiling studies of invasive breast carcinomas, including TNBC (10–14). Lehmann and colleagues characterized TNBC as a heterogeneous disease with seven molecular subtypes, including unstable, basal-like 1, basal-like 2, mesenchymal-like, mesenchymal stem-like (MSL), immunomodulatory, and luminal AR (LAR). The LAR subtype is similar to previously characterized molecular apocrine tumors (12, 13, 15) and its gene expression profile and chromatin-binding patterns mimic luminal, ER⁺ breast cancer, despite being ER-negative (11, 14). Within the TNBC molecular subtypes, LAR TNBC has the highest AR expression (16) and thus preclinical research has predominately focused on the efficacy of AR-targeted therapy using LAR cell lines as models of AR⁺ TNBC.

Our group and others have demonstrated that the LAR cell line MDA-MB-453 is sensitive to androgens *in vitro* (17, 18) and *in vivo* (17). Xenograft studies with AR antagonists have also demonstrated that LAR SUM185PE, CAL-148, and MDA-MB-453 cell lines are sensitive to bicalutamide (14) or enzalutamide (17). Although there are strong preclinical data to suggest that LAR TNBC subtypes may benefit from AR-targeted therapy, other TNBC molecular subtypes express AR and may also benefit from treatment with AR antagonists.

¹Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora, Colorado. ²Department of Medicine, Division of Oncology, University of Colorado Anschutz Medical Campus, Aurora, Colorado.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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A phase II trial of bicalutamide in ER⁻/PR⁻/AR⁺ metastatic breast cancer demonstrated a 19% clinical benefit rate (19), indicating that AR antagonists may be an effective targeted therapy for some patients with AR⁺ TNBC. A phase II trial (NCT01889238) of the newer generation AR antagonist enzalutamide, which blocks AR nuclear localization and is thus less likely to act as a partial agonist, is underway in TNBC. While the inclusion criteria for the current phase II trial of enzalutamide is 1% AR⁺ staining, most *in vitro* studies have focused on AR in LAR TNBC cell line models with very high AR expression and little is known about the role of AR or efficacy of enzalutamide in TNBC with lower AR expression. We hypothesized that non-LAR, AR⁺ TNBC may also critically depend on AR and could benefit from treatment with enzalutamide. Our study indicates that multiple subtypes of AR⁺ TNBC depend on AR for proliferation, migration, and invasion, and tumor growth *in vivo* and provides promising preclinical data on the efficacy of enzalutamide in TNBC with low AR expression.

Materials and Methods

Cell culture

All cell lines were authenticated by short tandem repeat analysis and tested negative for *Mycoplasma* in July 2014. Molecular subtypes of TNBC cell lines used in the present study were previously categorized by Lehmann and colleagues (14). SUM159PT cells were purchased from the University of Colorado Cancer Center Tissue Culture Core (Aurora, CO) in August 2013 and were grown in Ham/F-12 with 5% FBS, penicillin/streptomycin, hydrocortisone, insulin, HEPES, and L-glutamine supplementation. MDA-MB-231 (MDA231) cells were purchased from the ATCC in August 2008 and were grown in minimum essential media with 5% FBS, penicillin/streptomycin, HEPES, L-glutamine, nonessential amino acids, and insulin supplementation. HCC1806 cells were obtained from the laboratory of Dr. Haihua Gu in 2011 and propagated in RPMI-1640 with 10% FBS and penicillin/streptomycin. BT549 cells, purchased from the ATCC in 2008, were grown in RPMI-1640 with 10% FBS, penicillin/streptomycin, and insulin. All crystal violet assays were conducted in 5% charcoal-stripped serum to directly study the effect of DHT on cellular proliferation or transcription respectively. All other experiments were performed in full serum, as described above, with the exception of migration assays that were performed in serum-free conditions to prevent cellular proliferation.

SUM159PT-TGL and HCC1806-TGL cells were generated by stable retroviral transduction with a SFG-NES-TGL vector, encoding a triple fusion of thymidine kinase, GFP, and luciferase and sorted for GFP. SUM159PT, HCC1806, BT549, and MDA231 AR knockdown cells were generated by lentiviral transduction of shRNAs targeting AR (pMISSION VSV-G, Sigma Aldrich), including AR shRNA 3715 (shAR15) and AR shRNA 3717 (shAR17). Lentiviral transduction of pMISSION shRNA NEG (shNEG) was used as a nontargeting control. Plasmids were purchased from the University of Colorado Functional Genomics Core Facility.

Cellular assays and reagents

Cells were treated with 10 μ M enzalutamide (Medivation), 10 nmol/L DHT (Sigma-Aldrich), and 1 μ g/mL recombinant human amphiregulin (AREG; R&D Systems). A total of 10 μ M enzalutamide approximates the IC₅₀ of the 4 cell lines studied (data not shown) and is a clinically achievable concentration. Circulating plasma C_{max} values for enzalutamide and its

active metabolite (*N*-desmethyl enzalutamide) are 16.6 μ g/mL (23% CV) and 12.7 μ g/mL (30% CV), respectively (enzalutamide package insert Exposure Rationale), which is equivalent to approximately 60 μ M total active drug in plasma at steady state. Androgen concentrations have been previously examined in breast cancer (20), and intratumoral DHT concentrations (249 pg/g) were significantly higher than in blood. The DHT concentration of the present study is consistent with other *in vitro* studies of DHT in breast cancer (18, 21) and approximates levels of circulating testosterone in obese, postmenopausal women (22), as well as DHT levels in FBS used during routine tissue culture propagation (23).

Migration and invasion scratch wound assays were performed with or without BD Matrigel (BD Biosciences), respectively, per the manufacturer's instructions and scanned with the Incucyte ZOOM apparatus (Essen BioSciences). When an attractant was required for invasion, Transwell invasion assays were performed with BD BioCoat Matrigel Invasion Chambers (BD Biosciences) per the manufacturer's protocol. Caspase-3/7 fluorescent reagent (Essen BioSciences) was used at a dilution of 1:1,000 and normalized to cell count (apoptotic index), following the manufacturer's protocol, to assess apoptosis *in vitro*. The Amphiregulin Human ELISA Kit (Abcam) was used to measure extracellular AREG concentrations per the manufacturer's protocol.

For crystal violet assays, cells were fixed in 10% formalin, rinsed in PBS, and stained with 5% crystal violet. Crystal violet was then dissolved in 10% acetic acid and measured at 540 nm. MTS assays were performed with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol. Proliferation assays were also performed using the Incucyte ZOOM imaging system (Essen BioSciences). Soft agar assays were performed in 0.5% bottom and 0.25% top layer agar (Difco Agar Noble, BD Biosciences).

Tumor studies

Xenograft experiments were approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC protocol 83614(01)1E). All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. A total of 10⁶ SUM159PT-TGL or 500,000 HCC1806-TGL cells were mixed with Matrigel (BD Biosciences) and bilaterally injected into the mammary fat pads of female, athymic *nu/nu* mice (Taconic). Tumor burden was assessed by luciferase activity and caliper measurements [tumor volume was calculated as volume = (length \times width²)/2]. Once tumors were established, mice were randomized into groups based on the total tumor burden as measured by *in vivo* imaging. Mice were administered enzalutamide in their chow (~a 50 mg/kg daily dose). Enzalutamide was mixed with ground mouse chow (Research Diets Inc.) at 0.43 mg/g chow. The feed was irradiated and stored at 4°C before use. Mice in the control group received the same ground mouse chow but without enzalutamide. All mice were given free access to enzalutamide-formulated chow or control chow during the study period. Mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation, and the tumors and mammary glands were harvested.

Histology

Tissues were fixed in 10% neutral-buffered formalin, and tissue processing and paraffin embedding were performed by either the UCDenver Tissue Biobanking and Processing Core or the UCH

Anatomic Pathology Laboratory. Hematoxylin and eosin (H&E) stains were purchased from Anatech Ltd. and used per the manufacturer's instructions.

Archival formalin-fixed, paraffin-embedded primary breast tumors designated as hormone receptor-negative and $\text{HER2} \leq 10\%$ were collected under the Institutional Review Board protocol Molecular and Cellular Predictors of Breast Cancer (#10-0755) from 130 women diagnosed at Massachusetts General Hospital (Partners) between 1977 and 1993. Although many samples were originally defined as hormone receptor-negative by radioimmunoassay, all samples were reevaluated by immunohistochemistry and hormone receptor-negative was defined as $<1\%$ positive staining for ER and PR. Slides were immunostained for AR as described below and evaluated for the percentage and intensity of AR expression.

Immunohistochemistry

Slides were deparaffinized in a series of xylenes and ethanol, and antigens were heat retrieved in 10 mmol/L citrate buffer, pH 6.0. Antibodies used were AR clone 441 (Dakocytomation) and AREG (HPA008720, Sigma Aldrich). Envision horseradish peroxidase (Dakocytomation) was used for detection.

In situ hybridization

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining for apoptosis was performed using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection kit (Millipore), per the manufacturer's instructions.

Immunoblotting

Whole-cell protein extracts (50 μg) were denatured, separated on SDS-PAGE gels, and transferred to polyvinylidene fluoride membranes. After blocking in 3% BSA in TBS-Tween, membranes were probed overnight at 4°C. Primary antibodies used include: AR (PG-21, 1:500 dilution; EMD Millipore), TOPO1 (C-21, 1:100 dilution; Santa Cruz Biotechnology, Inc.), p44/42 MAPK (4695, 1:1,000; Cell Signaling Technology), phospho p44/42 MAPK (9101S, 1:500; Cell Signaling Technology), and α -tubulin (clone B-5-1-2, 1:30,000 dilution; Sigma Aldrich). Following secondary antibody incubation, results were detected using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer).

Cellular fractionation

Cellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology) as per the manufacturer's instructions.

Real-time quantitative PCR

RNA was isolated by TRIzol (Invitrogen), and cDNA was synthesized from 1 μg total RNA, using M-Mulv reverse transcriptase enzyme (Promega). SYBR green quantitative gene expression analysis was performed using the following primers: AREG forward, 5'-CGAACCACAAATACCTGGCTA-3' and AREG reverse, 5'-TCCATTTTGGCCTCCCTTTT-3'; β ACTIN forward, 5'-CTGTCCACCTCCAGCAGATG-3' and β ACTIN reverse, 5'-CGCAACTAAGTCATAGTCCGC-3'; and RPL13A forward, 5'-CCTGGAGGAGAGAGAGAAAGAGA-3' and RPL13A reverse, 5'-TTGAGGACCTCTGTGTATTTGTCAA-3'. Relative gene expression was calculated using the comparative cycle threshold method and values were normalized to β -ACTIN or RPL13A.

Statistical significance

Statistical significance was evaluated using a 2-tailed Student *t* test or ANOVA with GraphPad Prism software. $P \leq 0.05$ was considered statistically significant.

Results

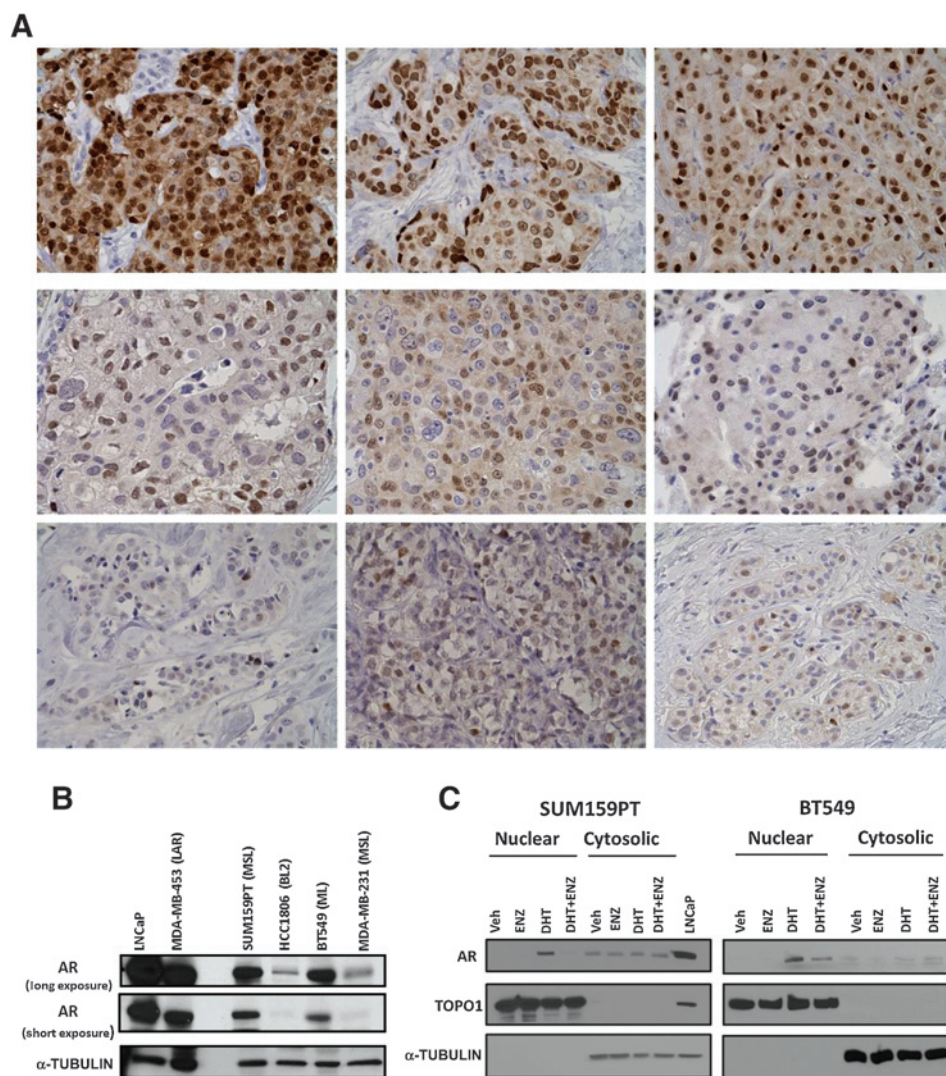
AR is expressed in 22% of TNBC patient tumors and in multiple molecular subtypes of TNBC

We examined 130 primary breast cancers designated as ER and PR negative, $\text{HER2} \leq 10\%$ positive [negative by College of American Pathology (CAP) and FDA criteria] for the presence of AR. In this group of tumors, 22% showed nuclear staining (range, 1%–100%). The presence of AR-positive tumor nuclei correlated with older patients using the Pearson Product Moment correlation ($r = 0.383$, $P < 0.0001$). There was a modest correlation between tumors from older patients with lower measures of proliferation (MIB-1: $r = -0.230$, $P = 0.0121$ and mitoses/10 hpf: $r = -0.204$, $P = 0.0255$). Representative images with a range of AR expression are displayed in Fig. 1A. Nuclear AR expression indicates that AR may be transcriptionally active in AR⁺ TNBC. Our findings are consistent with earlier studies which have reported AR protein expression in 12% to 36% of TNBC (6–9).

Previously, Lehmann and colleagues reported that TNBC is a heterogeneous disease with the highest AR mRNA and protein expression within the LAR molecular subtype of TNBC (14). However, AR is also expressed in cell lines representing the basal-like 1 and 2 (BL1, BL2), mesenchymal-like (ML), and MSL TNBC molecular subtypes (Fig. 1B) and may also present an opportunity for targeted therapy in these subtypes. In non-LAR TNBC cell lines, treatment with DHT increased nuclear localization of full-length AR, whereas enzalutamide, which blocks AR nuclear localization (17, 24, 25), inhibited this effect (Fig. 1C and Supplementary Fig. S1A). These findings demonstrate that AR nuclear localization is inhibited by enzalutamide in AR⁺ TNBC and that AR is expressed in cell lines representing multiple molecular subtypes of TNBC in addition to the LAR subtype.

AR inhibition decreases baseline proliferation and increases apoptosis in AR⁺ TNBC

AR inhibition was studied in 4 cell lines representing non-LAR TNBC subtypes including SUM159PT (MSL), HCC1806 (BL2), BT549 (ML), and MDA231 (MSL). By crystal violet staining, DHT increased baseline proliferation of the SUM159PT cell line and enzalutamide significantly decreased ligand-mediated and baseline proliferation in charcoal-stripped serum (Fig. 2A, $P < 0.01$). Interestingly, enzalutamide decreased baseline proliferation of HCC1806, BT549, and MDA231, but DHT did not increase proliferation in these cell lines when grown in charcoal-stripped serum. Enzalutamide also increased caspase-3/7 activity compared with vehicle control in SUM159PT, HCC1806, and BT549 (Fig. 2B, $P < 0.001$). Increased apoptosis was not observed in MDA231. In soft agar, enzalutamide significantly decreased colony formation compared with vehicle control in full serum conditions (Fig. 2C), suggesting that enzalutamide decreases anchorage-independent growth and may decrease tumorigenicity *in vivo*. Finally, SUM159PT cells were transduced with the AR^{F876L} mutation that confers resistance to enzalutamide. Compared with the parental cells, expression of AR^{F876L} prevented the growth-inhibitory effects of enzalutamide, indicating that the effects of enzalutamide are due to AR (Supplementary Fig. S1B).

**Figure 1.**

AR expression and nuclear localization in TNBC patient samples and cell lines. A, representative immunohistochemistry (IHC) of AR protein expression (brown) in TNBC patient samples. Photomicrographs represent a 400 \times magnification. B, Western blotting for AR expression in a panel of TNBC cell lines representing LAR, basal-like 2 (BL2), MSL, and mesenchymal-like (ML; ref. 14) subtypes of TNBC. The prostate cancer cell line LNCaP is shown as a positive control for AR. C, nuclear-cytoplasmic fractionation of TNBC cell lines grown in 5% charcoal-stripped serum for 48 hours and following a 3-hour treatment with vehicle control (Veh), enzalutamide (ENZ), and/or DHT. Topoisomerase I (TOPO1) is a loading control for the nuclear fraction and α -TUBULIN is a loading control for the cytosolic fraction.

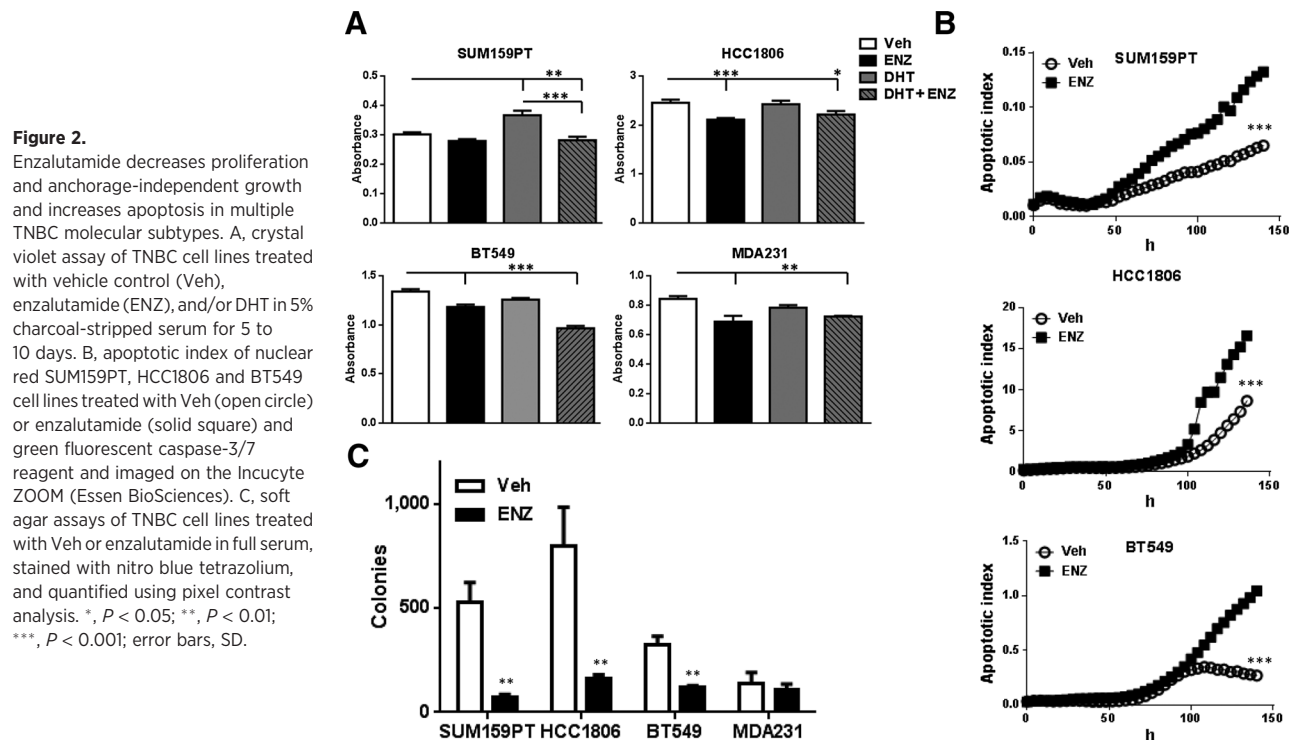
To confirm that the effects of enzalutamide are specific to AR inhibition, we examined the effects of shRNAs specifically targeting AR (shAR15, shAR17) compared with a nontargeting control (shNEG). Transduction of shRNAs targeting AR decreased full-length AR protein expression and significantly reduced proliferation in an MTS assay in non-LAR TNBC cell lines (Fig. 3A and B and Supplementary Fig. S1C). By crystal violet assay, AR knockdown significantly inhibited baseline and ligand-mediated proliferation of SUM159PT cells indicating that the shRNAs are effectively targeting AR (Fig. 3C). The SUM159PT cell line was chosen for this assay because DHT increases its baseline proliferation *in vitro*. AR knockdown also increased apoptosis in all 4 cell lines as measured by cleaved caspase-3 activity (Fig. 3D).

Enzalutamide decreases tumor viability *in vivo*

Luciferase-tagged SUM159PT-TGL cells, representing the MSL TNBC subtype, were bilaterally injected into the mammary fat pads of immunocompromised mice and treated with enzalutamide or vehicle control (Veh) following randomization when the tumors reached 50 mm³ (day -1, Supplementary Fig. S2A and S2B). Enzalutamide significantly decreased luciferase activity on

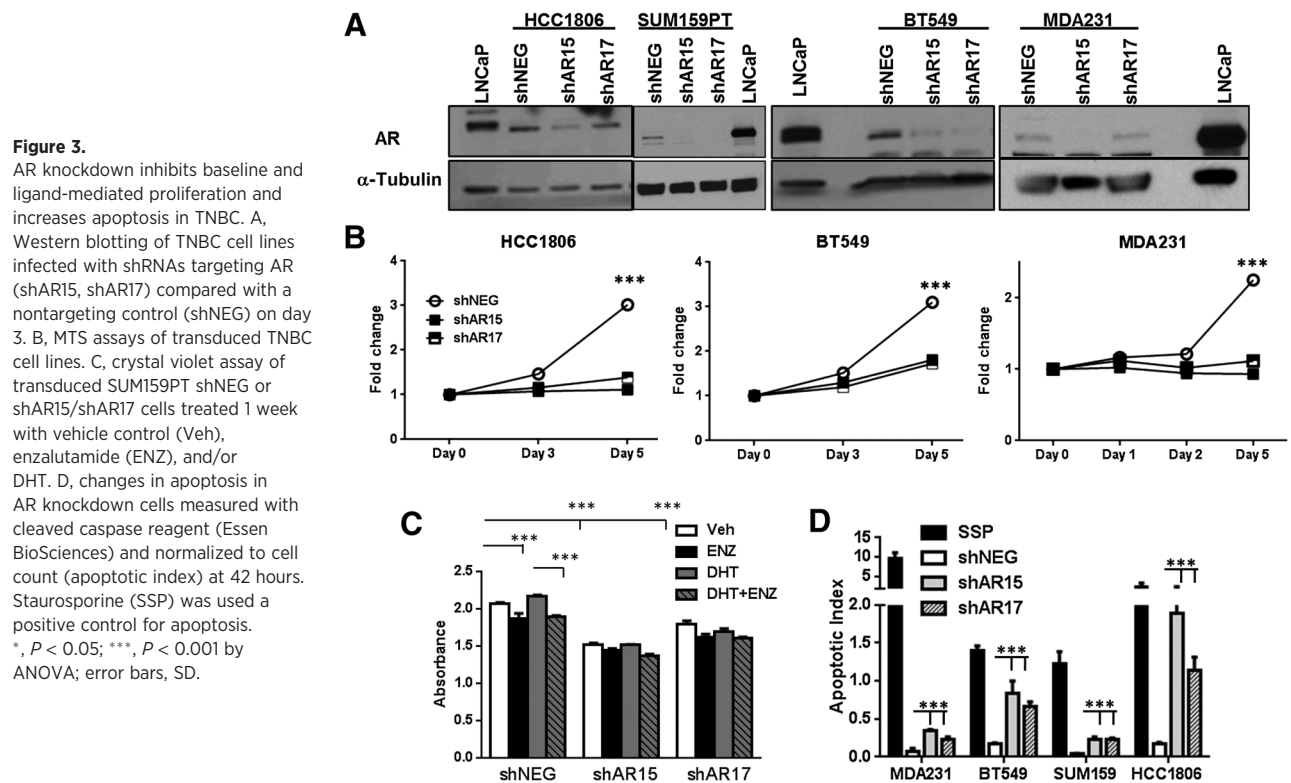
day 35 ($P = 0.008$, Fig. 4A–C). While no significant differences in caliper measurements or tumor weights were found between treatment groups (Supplementary Fig. S3A–S3C), H&E staining demonstrated that the median percentage of necrotic tumor was 90% in the enzalutamide treatment group compared with 10% in Veh xenografts ($P = 0.009$, Fig. 4D). The percentage of necrotic tissue of H&E-stained sections was determined in a blinded fashion by a board-certified pathologist (P. Jedlicka). Enzalutamide-treated xenografts also exhibited a 4-fold increase in TUNEL staining ($P = 0.04$, Fig. 4E) and a 2-fold decrease in AR score (score = intensity range 0 to 3 \times percent nuclear positivity, $P = 0.07$, Fig. 4F) compared with vehicle-treated controls.

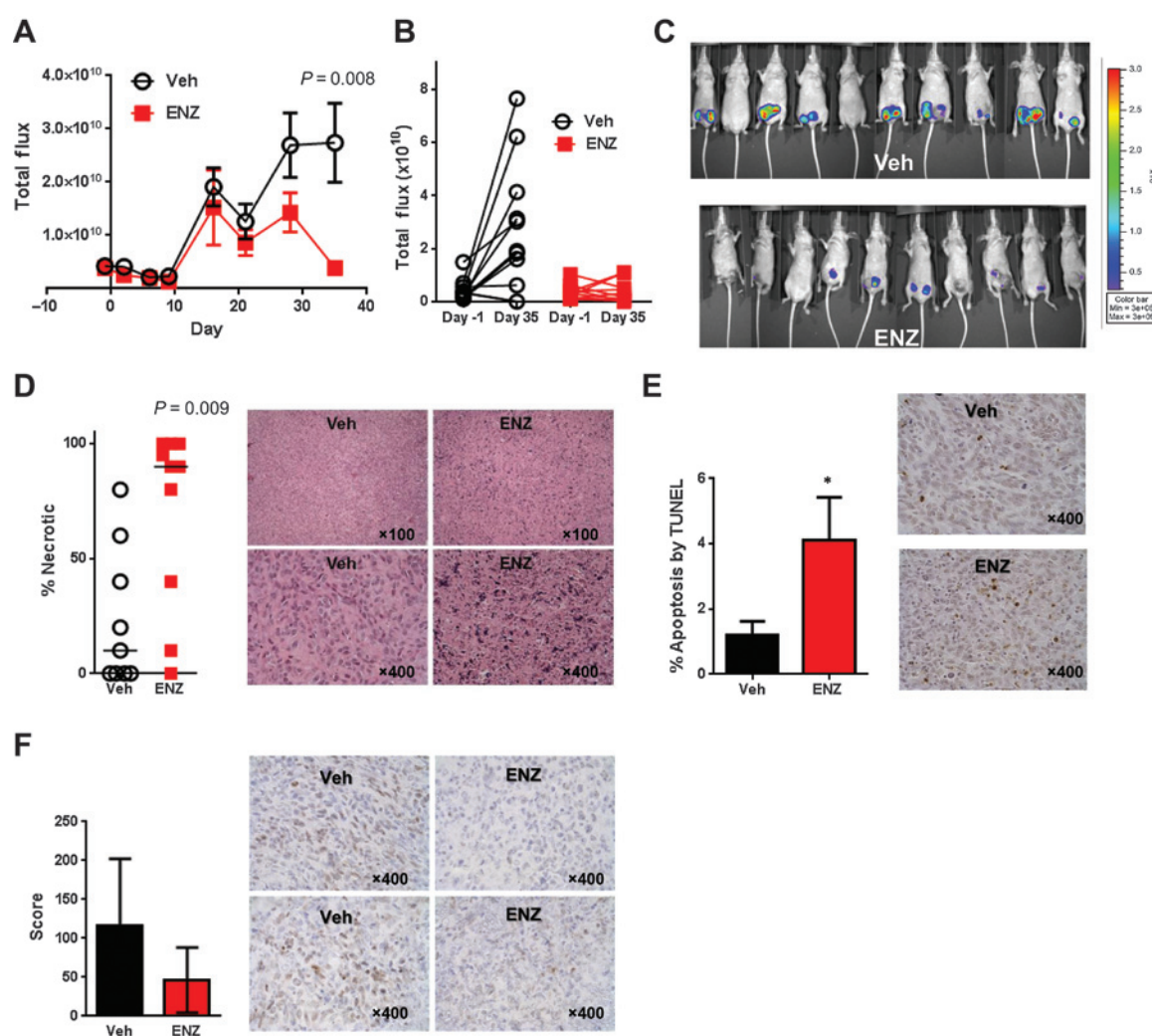
As in the SUM159PT xenograft study, luciferase-tagged HCC1806-TGL cells, which represent the BL2 TNBC subtype, were bilaterally injected into the mammary fat pads of immunocompromised mice and treated with enzalutamide or vehicle following randomization (Supplementary Fig. S2C and S2D). Enzalutamide significantly decreased luciferase activity on days 10 and 14 ($P < 0.001$, Supplementary Fig. S4A–S4C). HCC1806 xenografts grew at a faster rate than SUM159PT xenografts, resulting in early termination of the study on day 14 and a high



degree of necrosis in both treatment groups. However, by H&E staining, enzalutamide-treated xenografts displayed increased necrosis (Supplementary Fig. S4D). Enzalutamide-treated xeno-

grafts also exhibited increased TUNEL staining compared with vehicle-treated controls ($P = 0.04$, Supplementary Fig. S4E). No significant differences in caliper measurements or tumor weights



**Figure 4.**

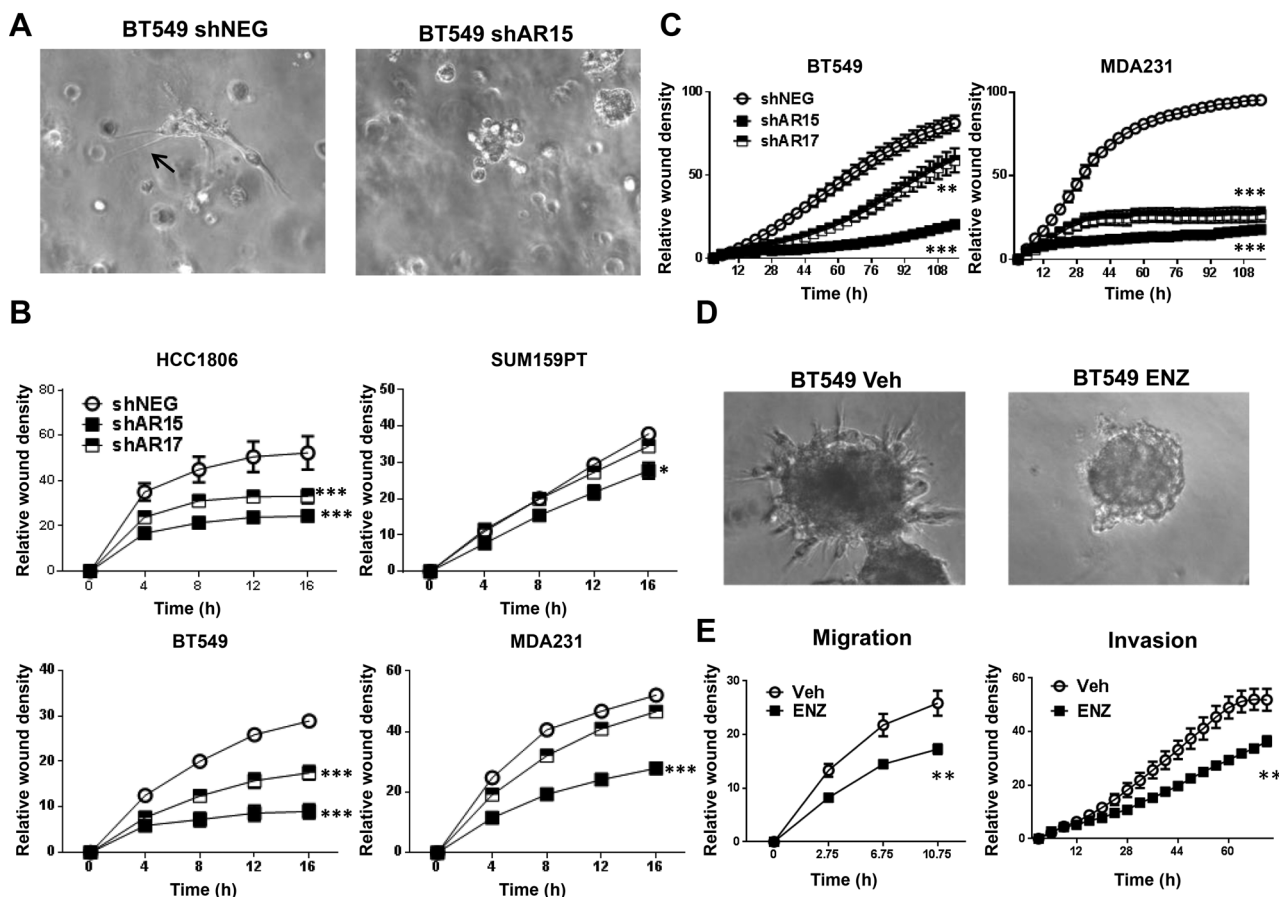
Enzalutamide (ENZ) decreases cellular viability and increases necrosis and apoptosis in SUM159PT xenografts. A, total flux growth curve of SUM159PT nude mice xenografts. Mice were randomized at day -1 and treatment was initiated on day 0. P value represents a 2-tailed t test comparing total flux between groups on day 35 and error bars represent SEM. B, change in total flux between randomization and day 35, by mouse. C, luminescent overlay of Veh and enzalutamide-treated mice. D, percent necrotic tissue by H&E staining. Horizontal bars represent median percentage necrotic tissue. P value represents a 2-tailed t test comparing percent necrosis between groups on day 35. Photomicrographs depict examples of tumor xenograft H&E staining showing viable tumor (Veh) and necrotic tumor (ENZ). E, TUNEL staining for apoptosis. Photomicrographs depict examples of TUNEL staining. F, AR nuclear score (score = intensity range 0 to 3 \times % positive) by IHC. Photomicrographs depict examples of AR staining in SUM159PT xenografts. $*$, $P < 0.05$; error bars, SEM.

were found between treatment groups (Supplementary Fig. S3D–S3F). In summary, our results show that enzalutamide decreases cellular viability while increasing necrosis and apoptosis *in vivo* in 2 non-LAR molecular subtypes of TNBC in addition to the LAR MDA-MB-453 cell line previously reported (17).

AR inhibition alters cellular morphology and decreases migration and invasion

AR knockdown altered cellular morphology of BT549 and MDA231 cells in 3D Matrigel (BD Biosciences) culture from stellate to round (Fig. 5A). In a scratch wound assay, AR knockdown significantly decreased migration compared with a nontargeting control in 4 AR⁺ non-LAR cell lines (Fig. 5B). Scratch wound assays were conducted in serum-starved, attractant-free conditions and over a short time course to minimize potential confounding effects

of AR knockdown on cell proliferation. MDA231 and BT549 cell lines invade through Matrigel without an attractant and AR knockdown in these cell lines inhibited invasion (Fig. 5C). Changes in cellular morphology and decreased migration and invasion were next examined in BT549 cells treated with enzalutamide. In 3D Matrigel, cellular morphology was altered from predominately stellate to predominately round (Fig. 5D), and migration (Fig. 5E, left) and invasion (Fig. 5E, right) were significantly inhibited by enzalutamide. In identical serum-starved conditions, BT549 control wells treated with enzalutamide and cleaved caspase reporter (Essen BioSciences) exhibited no changes in proliferation or apoptosis (Supplementary Fig. S5), demonstrating that AR influences migration independently of proliferation or apoptosis. At this concentration of enzalutamide, no significant changes in migration were observed in other non-LAR cell lines tested.

**Figure 5.**

AR inhibition decreases migration and invasion of TNBC cells. A, cellular morphology (200 \times) of BT549 cells transduced with a nontargeting control (shNEG) compared with a shRNA targeting AR (shAR15) in 3D Matrigel culture. Arrow, stellate cellular morphology. B, migration scratch wound assay of TNBC cell lines with AR knockdown under serum-starved conditions. C, scratch wound assay of TNBC AR knockdown cell lines invading through Matrigel. D, changes in cellular morphology of BT549 cells treated with vehicle (Veh) or enzalutamide (ENZ) in 3D Matrigel culture (200 \times). E, migration (left) and invasion (right) assays of BT549 cells treated with or without enzalutamide. **, $P < 0.01$; ***, $P < 0.001$ by the t test at the final time point.

Amphiregulin is regulated by AR in TNBC and rescues decreased proliferation and migration associated with AR inhibition

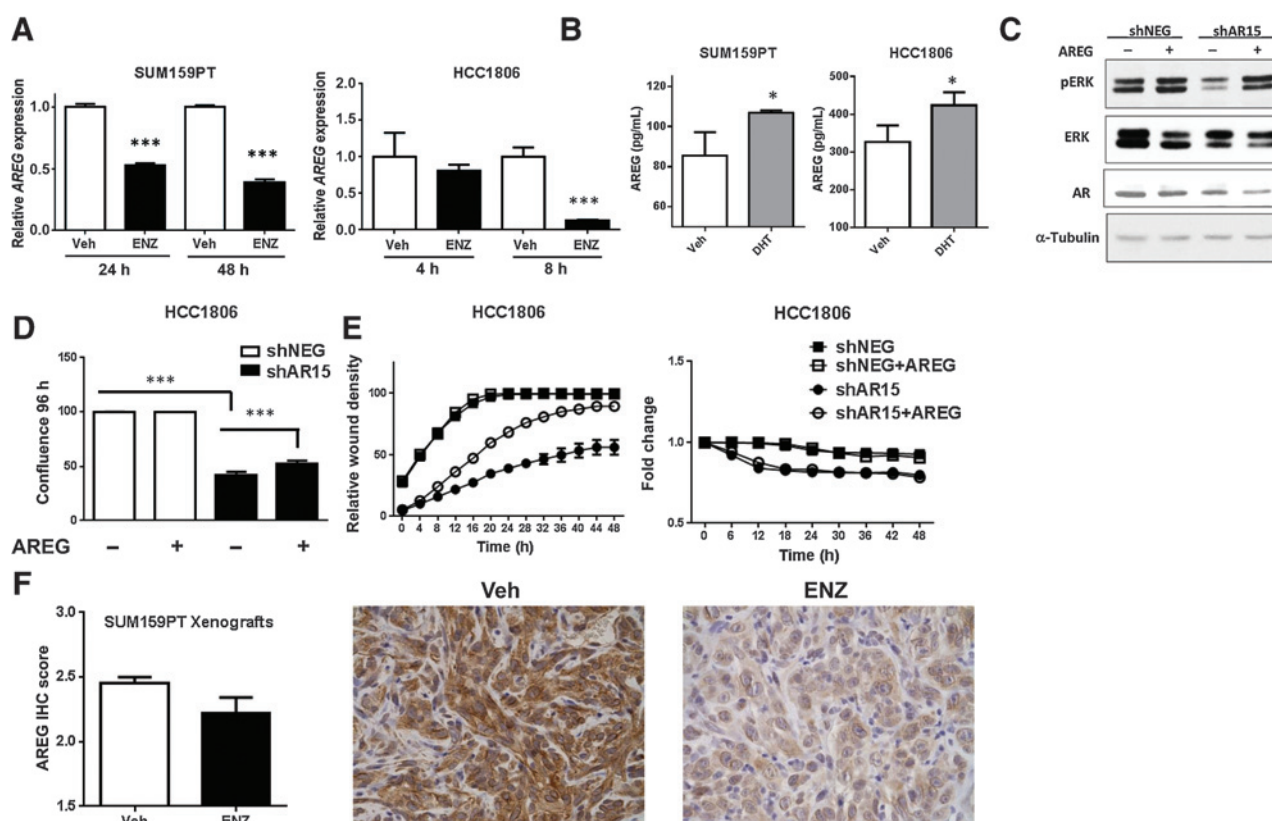
By microarray and AR chromatin immunoprecipitation of an immortalized human prostate epithelial cell line, Bolton and colleagues identified amphiregulin (AREG) as an AR-regulated gene (26). AREG is required for mammary ductal morphogenesis and is the predominant EGF receptor (EGFR) ligand during mammary gland development (27). To date, AR is not known to regulate AREG in breast cancer or normal breast tissue. However, within TNBC, AR expression correlates with activated EGFR (28). We thus hypothesized that AR may regulate AREG in TNBC.

By quantitative real-time PCR (qRT-PCR), treatment with enzalutamide decreased AREG mRNA expression by 2-fold in SUM159PT and 4-fold in HCC1806 ($P < 0.001$, Fig. 6A). At the protein level, treatment with DHT significantly increased secreted AREG by ELISA in both SUM159PT and HCC1806 ($P < 0.05$, Fig. 6B). EGFR activation by AREG induces multiple downstream signaling pathways including MAPK (16). Compared with nontargeting controls, AR knockdown decreased endogenous phosphorylation of ERK, and exogenous AREG rescued this effect in HCC1806 (Fig. 6C). These results were recapitulated in the SUM159PT cell line (Supplementary Fig. S6A).

Given our data suggesting that AR regulates AREG which activates the MAPK signaling pathway with key roles in proliferation, migration, and invasion (29), we next tested whether exogenous AREG would rescue the phenotypes associated with AR inhibition. As in Fig. 3B, AR knockdown significantly inhibited proliferation of HCC1806 compared with nontargeting controls, and the addition of exogenous AREG partially rescued this effect ($P < 0.0001$, Fig. 6D). Similarly, AR knockdown decreased migration of HCC1806 cells (as in Fig. 5B) and exogenous AREG partially rescued this effect (Fig. 6E, left) without altering proliferation in these serum-free conditions (Fig. 6E, right). Exogenous AREG also partially rescued proliferation and invasion in SUM159PT (Supplementary Fig. S6B and S6C). Enzalutamide-treated SUM159PT xenografts displayed decreased AREG expression compared with vehicle controls (Fig. 6F). Together, these data indicate that AR regulation of AREG is one mechanism by which AR effects proliferation, migration, and invasion in AR⁺ TNBC.

Discussion

Compared to patients with non-TNBC, patients with TNBC with residual disease following chemotherapy have a significantly

**Figure 6.**

AR regulation of amphiregulin mediates baseline proliferation and migration of TNBC. A, quantitative real-time PCR (qRT-PCR) for amphiregulin (AREG) in SUM159PT cells and HCC1806 cells treated with enzalutamide (ENZ) in full serum. B, ELISA for extracellular AREG in SUM159PT and HCC1806 cell lines treated with vehicle (Veh) or dihydrotestosterone (DHT) for 48 and 72 hours, respectively. C, Western blotting of HCC1806 shNEG and shAR15 cells treated for 30 minutes with exogenous human recombinant AREG. D, proliferation assay of HCC1806 shNEG and shAR15 cells in the absence or presence of exogenous AREG. E, migration (left) and proliferation (right) assays of HCC1806 cells treated with or without exogenous AREG in identical, serum-starved conditions. F, AREG protein expression by IHC in SUM159PT xenografts. $P = 0.04$ using a 1-tailed t test. Photomicrographs depict representative AREG staining (400 \times). *, $P < 0.05$; ***, $P < 0.001$ by the t test.

worse overall survival (2). The poor prognosis of patients with TNBC is due, in part, to a lack of effective targeted therapy. However, AR is expressed in up to a third of patients with TNBC (6–9) and represents an opportunity for targeted therapy. Indeed, if AR-targeted therapy is effective in AR⁺ TNBC, it would represent the first effective targeted therapy for this aggressive breast cancer subtype and would greatly benefit this population of women. Previous studies focused on the role of AR in the high AR-expressing, LAR molecular subtype of TNBC and found that this subtype was responsive to bicalutamide, whereas the non-LAR subtypes were less responsive or nonresponsive (14). In contrast, we find that multiple non-LAR subtypes (mesenchymal-like, MSL, and basal-like 2) with relatively low AR expression critically depend on AR for proliferation, migration, and invasion and that even those previously found to be resistant to bicalutamide are sensitive to the new-generation anti-androgen enzalutamide *in vitro* and *in vivo*.

In TNBC cell lines representing the "mesenchymal-like," "mesenchymal stem-like," and "basal-like" molecular subtypes (14), pharmacologic inhibition of AR with enzalutamide and AR knockdown decreased proliferation and anchorage-independent growth and increased apoptosis. Thus, AR may be required for optimal baseline proliferation even though DHT does not increase proliferation in all AR⁺ TNBC cell lines. The discordance

between baseline inhibition and lack of ligand-mediated proliferation in some cell lines may indicate that the mechanism by which AR mediates proliferation is nontranscriptional or less ligand-dependent in the non-LAR cell lines. Interestingly, MDA231 cells were less sensitive to enzalutamide by soft agar and caspase-3/7 assays and a recent study suggests that this may be due to expression of AR variant 3, which lacks the ligand-binding domain, in this cell line (30).

Decreased viability and increased apoptosis by AR inhibition *in vitro* was recapitulated in SUM159PT and HCC1806 xenografts in nude mice. Although other groups have suggested that AR inhibition could promote survival through activation of PI3K signaling (31), enzalutamide significantly decreased survival in both wild-type (HCC1806) and PIK3CA-mutant (SUM159PT) cell lines. Sensitivity of SUM159PT xenografts to enzalutamide contrasts previous work demonstrating that bicalutamide did not inhibit tumor volume (14). The discrepancy in results may be due to differences in the mechanisms of action of the two AR antagonists. Bicalutamide permits AR nuclear localization and binding to chromatin, recruiting corepressors rather than coactivators, whereas enzalutamide inhibits nuclear localization and DNA binding (32). Bicalutamide has partial agonist effects in prostate cancer (33) and thus may also have partial agonist effects in TNBC. However, it should be noted that enzalutamide

significantly increased tumor necrosis but did not decrease tumor volume according to caliper measurements. Thus, an increase in necrosis may not have been apparent in the bicalutamide study by measurement of tumor volume alone.

A phase II clinical trial of bicalutamide in AR⁺/ER⁻/PR⁻ metastatic breast cancer reported a 19% clinical benefit rate and a 12-week longer median progression-free survival (19). Of note, the study included HER2-amplified patients and required 10% AR-positive staining for trial eligibility. However, bicalutamide has partial agonist effects (33) and patients with prostate cancer who acquire resistance to bicalutamide often respond to enzalutamide (34), suggesting that enzalutamide may be a more effective antagonist in TNBC. The results of the present study are promising and timely as a phase II clinical trial is currently testing the efficacy of enzalutamide in AR⁺ TNBC (NCT01889238). Our finding that non-LAR subtypes also critically depend on AR indicates that patients with relatively low AR expression may also benefit from AR-targeted therapy. Indeed, the trial has recently expanded patient eligibility to 1% AR⁺ staining, which may improve the number of patients eligible for treatment.

In vitro, AR inhibition altered cellular morphology and decreased migration and invasion suggesting that AR⁺ TNBC is also dependent on AR for these functions. Extensive evidence suggests that advanced, metastatic prostate cancer is causally related to continued AR activation (5), and recent prostate and bladder cancer studies demonstrate that AR regulates multiple metastasis-promoting genes (35–37). In breast cancer, initial surgically resected breast cancer metastases retain nuclear AR expression as in the primary tumor (38). Interestingly, breast cancer metastases, including those in patients with TNBC, also have significantly increased AR phosphorylation (39), indicative of active receptors.

Multiple studies have demonstrated that AR expression is associated with an overall favorable prognosis in breast cancer including the TNBC subtype (8, 40–42). However, this is not surprising because like ER, AR is indicative of a more well-differentiated form of the disease but may still drive tumor growth and therefore serve as a rational therapeutic target. High AR expression may be indicative of a more luminal, well-differentiated, less aggressive tumor, and this confers a good prognosis. Future studies are needed to further characterize the role of AR in breast cancer metastasis and determine if AR-targeted therapy will reduce metastatic burden in preclinical models of TNBC.

Treatment with AREG, an EGFR ligand with critical roles in normal mammary gland development, partially rescued decreased proliferation, migration, and invasion resulting from AR knockdown. Thus, AR regulation of AREG may be one mechanism by which AR mediates these critical functions and AR antagonists may also indirectly target EGFR signaling. Recent clinical trials demonstrate that treatment with the anti-EGFR

antibody cetuximab in addition to chemotherapy may benefit patients with metastatic breast cancer (43, 44). *In vitro* data suggest that combined AR antagonist and EGFR or ERK1/2 inhibitors may be effective in TNBC (28). Exogenous AREG only partially rescued phenotypes associated with AR inhibition indicating that additional AR-regulated genes are likely involved in these phenotypes.

Although extensive genomic studies to identify novel therapeutic strategies have expanded our knowledge of the diverse molecular biology of TNBC, there are currently no effective targeted therapies for patients with TNBC. The present study demonstrates that multiple molecular subtypes of TNBC depend on AR for critical cancer phenotypes including viability, migration, and invasion. Despite heterogeneity among tumors, hormone receptor-targeted therapies have greatly improved the prognosis of multiple hormone-related malignancies and exploiting AR dependence with AR-targeted therapies may ultimately improve TNBC patient prognosis.

Disclosure of Potential Conflicts of Interest

A. Elias has received other commercial research support from Medivation and Astellas. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V.N. Barton, M.A. Gordon, N.S. Spoelstra, R.E. Heinz, J.K. Richer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.N. Barton, P. Jedlicka, B.M. Jacobsen, J.K. Richer
Writing, review, and/or revision of the manuscript: V.N. Barton, N.C. D'Amato, M.A. Gordon, N.S. Spoelstra, A. Elias, B.M. Jacobsen, J.K. Richer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.N. Barton, H.T. Lind
Other (animal husbandry): B. L. Babbs

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Molecular Cancer Therapeutics

Multiple Molecular Subtypes of Triple-Negative Breast Cancer Critically Rely on Androgen Receptor and Respond to Enzalutamide *In Vivo*

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Androgen Receptor Biology in Triple Negative Breast Cancer: a Case for Classification as AR+ or Quadruple Negative Disease

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Abstract Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype that lacks estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) amplification. Due to the absence of these receptors, TNBC does not respond to traditional endocrine or HER2-targeted therapies that improve patient prognosis in other breast cancer subtypes. TNBC has a poor prognosis, and currently, there are no effective targeted therapies. Some TNBC tumors express androgen receptor (AR) and may benefit from AR-targeted therapies. Here, we review the literature on AR in TNBC and propose that TNBC be further subclassified as either AR+ TNBC or quadruple negative breast cancer since targeting AR may represent a viable therapeutic option for a subset of TNBC.

Introduction

Triple negative breast cancer (TNBC), a term first published in 2005 [1], is defined by negative clinical testing for estrogen receptor (ER α), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) amplification. Because it lacks these receptors, TNBC is unresponsive to traditional estrogen pathway-directed endocrine therapies or HER2-targeting therapies. Currently, there are no targeted therapies for TNBC, and chemotherapy remains the best therapeutic option. However, upon recurrence of chemoresistant disease, effective

therapeutic options are limited. Indeed, TNBC constitutes 15–20 % of newly diagnosed invasive breast carcinomas and has the lowest 5-year survival rate compared to other breast cancer (BC) subtypes. A study of over 1600 women found that women with TNBC have a peak risk of recurrence between 1 and 3 years, an increased likelihood of distal recurrence, and a majority of deaths occurring in the first 5 years compared to other BC subtypes [2]. Overall BC outcomes have greatly improved as a result of early diagnosis, however TNBC often presents between mammograms [2].

Although TNBC lacks hormone receptors traditionally associated with BC, both molecular and immunohistochemical analyses demonstrate that a subset of TNBC expresses the androgen receptor (AR). Emerging data suggest that AR significantly influences breast cancer gene expression profiles and affects tumorigenic properties of TNBC. Development of new generation anti-androgens for the treatment of prostate cancer has led to renewed interest in hormonal therapy targeting AR in the subset of AR+ TNBC and constitutes a novel therapeutic option that could improve prognosis with few side effects. Herein, we discuss the role of AR in the biology of TNBC in preclinical models and review the clinical data on the efficacy of targeting AR in TNBC. Based on these data, we propose that testing for AR should become the standard clinical practice and that TNBC be further defined as either AR+ TNBC or “quadruple negative” disease to emphasize the utility of AR as a viable therapeutic target in AR+ breast cancer.

AR Expression in TNBC

Multiple studies report nuclear AR expression in TNBC patient specimens [3–11]. Importantly, nuclear AR staining is indicative of active receptors, since AR translocates to the nucleus upon binding ligand. The percentage of TNBC with

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nuclear AR expression (as detected by IHC) ranges from 12–55 % depending on the study. The large range of AR expression may reflect differences in AR antibodies used, antigen retrieval methods, and the criteria for positive AR expression (either 1 or 10 % positivity). In two prospectively conducted clinical trials, rates of AR+ disease ranged from 12 % (AR \geq 10 %, DAKO) [10] to 55 % (AR \geq 10 %, DAKO and Ventana) [11]. Studies comparing AR expression in primary versus metastatic disease found that AR is frequently retained in metastatic samples from patients with AR+ primary tumors [12, 13]. Reports of AR expression in TNBC patient samples are summarized in Table 1. To date, there is no standardized, optimized assay to assess AR expression in TNBC. As we move toward classification of AR+ and quadruple negative disease, standardization of AR IHC is of high priority. Interestingly, the percent of AR+ cells alone may not be sufficient to identify patients who will benefit from AR-targeted therapy. For example, the genomic biomarker reported by Traina et al. predicted patients who would benefit from enzalutamide (Enza) despite having low AR expression by IHC [14]. AR expression alone was not significantly associated with patient outcome. Thus, a combination of AR expression by IHC \geq 1 % and genomic biomarker expression may best identify the cohort of AR+ TNBC patients who will benefit from AR-targeted therapy.

Across all subtypes of BC, AR expression is associated with a better overall survival and disease-free survival irrespective of co-expression of ER α in breast cancer [15]. Within TNBC, the prognostic significance of AR is controversial (reviewed in [16]) as AR expression has been associated with both a good and bad prognosis in multiple studies. As AR is expressed in normal mammary epithelial cells (see primary data in [17]), it is likely associated with a more well-differentiated, less aggressive tumor. Indeed, AR+ TNBC has a lower Ki-67 index than AR– TNBC [3] and may therefore be less responsive to chemotherapy. This is supported by the lower pathologic complete response (pCR) rate following chemotherapy of TNBC tumors with gene expression profiles enriched in AR signaling pathways [18] compared to other

TNBC subtypes. Just as ER α confers a good prognosis, but is an effective therapeutic target in ER+ breast cancer [19–22], AR may similarly confer an overall better prognosis since it is expressed in slower growing tumors, yet serve as an effective therapeutic target in a subset of TNBC that are dependent on or driven by this receptor.

In TNBC tumor cohorts, studies identified correlations between AR protein positivity and other proteins of biological importance. AR protein expression is positively correlated with aldehyde dehydrogenase 1 (ALDH1) [23], an enzyme associated with stem-like cells in breast cancer, as well as 17 β -hydroxysteroid dehydrogenase 5 (17 β HSD5) and 5 α -reductase type 1 (5 α R1), enzymes involved in androgen synthesis [24]. Conversely, AR protein expression is negatively correlated with the L1 cell adhesion molecule (L1CAM) [25] and tight junction protein Claudin 4 (CLDN4) [26]. These studies raise the interesting possibility that AR may promote a stem-like or mesenchymal phenotype in TNBC, an observation consistent with the lower Ki67 staining associated with AR+ TNBC.

TNBC Lacks AR Mutations but Expresses AR Splice Variants

The Cancer Genome Atlas (TCGA) sequencing data revealed 2 patients with single missense mutations among 93 TNBCs analyzed (2.2 % mutation rate). One mutation, D865E, localized to the ligand-binding domain of AR and the other, L638M, localized to a domain for which a specific function have not been ascribed. Neither mutation contributes to a reported or predicted functional alteration of AR. By FISH analysis of 99 BC and normal breast tissues, AR gene amplification was not found [27]. As tissue from clinical trials targeting AR in TNBC becomes available, amplification or mutations in AR may be identified as a result of AR-targeted therapy.

AR splice variant mRNA is expressed in BC tissues and cell lines [28, 29]. An AR variant without exon 3 (Δ 3AR) that lacks the second zinc finger of the DNA binding domain was

Table 1 AR protein expression in TNBC tumor specimens

% AR+	N	% Nuclear AR	Antibody	Reference
12 %	43	>10 %	AR441, Dako	[10]
22 %	130	\geq 1 %	AR441, Dako	[3]
30 %	135	>10 %	AR27, Novocastra	[4]
32 %	239 ^a	>10 %	AR441, Dako	[5]
33 %	83	\geq 1 %	M3562, Dako	[6]
36 %	50	\geq 1 %	AR441, Dako	[7]
38 %	699	\geq 1 %	AR27, NCL-AR-318	[8]
53 %	158	\geq 10 %	AR441, Dako	[9]
55 %	203	\geq 10 %	AR441, Dako and Ventana SP107	[11]

^a Defined as basal-like

identified in BC, but not normal, tissues [28]. Hu et al. also reported low levels of AR variant expression in normal breast tissue with some expression of AR variant AR45, which is truncated at the N-terminus [29]. AR45 lacks exon 1 and is associated with decreased AR activity in prostate cancer [30]. TNBC cell lines MDA-MB-453 and MDA-MB-231 express AR45 and AR variant 3, respectively [29]. AR variant 3, which lacks a ligand binding domain, is ligand independent and constitutively active in prostate cancer [31]. Additional studies are needed to verify AR variant expression in TNBC tumor specimens at the protein level, particularly after treatment with AR-targeted therapies.

AR and Hormone-Regulated Genes Define a Subtype of TNBC

Molecular profiling experiments have redefined BC subtypes to take into account the strong effects of AR, which is expressed across BC subtypes [32–34]. In a study examining mRNA expression patterns in over 500 breast tumors representing all BC subtypes, Guedj et al. identified an AR-regulated gene cluster as one of three key gene clusters that define global BC expression patterns [32]. The AR-associated gene cluster included a molecular apocrine AR+/PR-/ER- subgroup (mApo, comprised of tumors with and without HER2 amplification) as well as a basal-like AR-/ER-/PR- subgroup (BasL) [32]. Whereas the mApo subgroup was enriched in immune signaling pathways, the BasL subgroup was enriched in DNA replication and repair signaling. The expression profile of the mApo, AR+/ER-/PR- subgroup is similar to the previously described “molecular apocrine” group of tumors enriched with expression of ER-regulated genes, despite being ER- [33, 34]. It is proposed that in these tumors, AR promotes transcription of many of these classical ER-regulated genes.

More recently, expression analysis of over 500 TNBC patient samples identified significant heterogeneity that includes a subtype with a “luminal AR” gene signature as well as “basal-like 1,” “basal-like 2,” “immunomodulatory,” “mesenchymal-like,” and “mesenchymal stem-like” subtypes [35]. The luminal AR (LAR) subtype was so termed because it has a molecular expression profile similar to ER+ breast cancers. Gene ontologies defining the LAR subtype were enriched in hormonally regulated pathways including steroid synthesis and androgen/estrogen metabolism. Importantly, the LAR subtype was associated with a poor patient prognosis suggesting that this subtype may be less responsive to chemotherapy. Masuda and colleagues also compared pCR rates following neoadjuvant chemotherapy of the TNBC subtypes and found that the pCR rates differed by subtype [18]. The basal-like 1 subtype had the highest pCR rate whereas the LAR and basal-like 2 subtypes had the lowest pCR rates. A low Ki-67

index associated with reduced proliferation may account for the low pCR rate among LAR tumors.

Subsequent TNBC profiling studies corroborated the existence of a LAR TNBC subtype. Recently, Burstein and colleagues examined 198 TNBC tumors and identified four distinct subtypes including LAR with the subtype specific marker mucin 1 (MUC1) [36]. Similarly, in a study of 107 TNBC, Jezequel et al. identified three subtypes including LAR [37]. Identification of an AR signaling-based subgroup of TNBC tumors by multiple independent studies further supports the need to distinguish quadruple negative breast cancer and AR+ disease in the clinic to optimize therapy and improve patient outcomes.

Molecular Biology of AR in the Luminal AR TNBC Subtype

LAR tumors exhibit a tenfold higher AR protein expression than non-LAR subtypes [35]. Xenograft experiments with LAR TNBC cell lines demonstrated greater sensitivity to the AR antagonist bicalutamide compared to non-LAR xenografts. However, bicalutamide is known to have partial agonist effects [38], and prostate cancer patients who acquire resistance to bicalutamide are often responsive to the next generation antagonist Enza, which has higher affinity for AR and attenuates DNA binding [39], while bicalutamide allows nuclear translocation and DNA binding. Indeed, non-LAR, AR+ SUM159PT and HCC1806 xenografts that were insensitive to bicalutamide [35] were sensitive to Enza [3], suggesting that response to pure AR antagonists may not be limited to the LAR TNBC subtype.

LAR cell lines have a high frequency of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations [40]. The combination of PI3K/mTOR and AR inhibitors showed additive growth inhibitory effects in vitro. Additive effects of bicalutamide with PI3K inhibitor GDC-0941 or PI3K/mTOR inhibitor GDC-0980 were also observed in MDA-MB-453 and CAL-148 LAR xenografts [40]. In a comparison of global DNA-binding events, Robinson et al. found that AR binding in the LAR TNBC cell line MDA-MB-453 was more similar to ER binding in the ER+ breast cancer cell line (MCF7) than AR binding in a prostate cancer cell line (LNCaP) [41]. Thus, in the absence of ER, AR may function similarly to ER, accounting for the luminal transcriptome of this subtype. AR binding further required the forkhead box protein A1 (FOXA1) pioneer factor.

Most research on the LAR subtype has utilized the MDA-MB-453 cell line. This cell line is classified as LAR by gene expression profiling [35], is strongly positive for AR by western blot and IHC, and is very responsive to new anti-androgens [42, 43]. However there is discrepancy with regard to HER2 amplification and overexpression status, rendering

the status of this cell line as true TNBC debatable. Reported HER2 gene amplification rates as measured by FISH range from 1.08–2.39 [44–47]. Our own internal testing of MDA-MB-453 from two separate sources revealed FISH scores varying between 1.43 and 2.83. Clinically, positive HER2 amplification is defined as a FISH ratio (HER2-to-CEP17) higher than 2.2 or HER2 gene copy greater than 6 [48]. Studies have also shown that some MDA-MB-453 cell lines are innately non-responsive to trastuzumab [49, 50]. In summary, amplification of HER2 is present in some MDA-MB-453 cell lines and reliance on the HER2 receptor is likely dependent on the HER2 amplification levels of MDA-MB-453 cell lines that have drifted in various laboratories. Thus, in order to classify MDA-MB-453 as a TNBC line, research groups should test their MDA-MB-453 cell lines for HER2 amplification status and perhaps this line may represent a TNBC that is HER2-enriched [51]. MDA-MB-453 also has an AR mutation in the ligand binding domain that decreases receptor activity [52] and may render it a unique model of AR+TNBC, although it has high AR protein levels and is very responsive to anti-androgens in vitro and in vivo [42]. Further studies using additional LAR cell lines and patient-derived xenografts may facilitate a better characterization of the biology of this TNBC subtype.

Molecular Biology of AR in Non-Luminal AR TNBC Subtypes

Although AR is most highly expressed in LAR tumors, AR is expressed in non-LAR subtypes [3, 53]. Cell lines representing non-LAR and AR+ TNBC subtypes, including mesenchymal stem-like, mesenchymal-like and basal-like, exhibit decreased anchorage-independent growth when treated with the AR antagonist Enza [3]. AR knockdown and treatment with Enza decreased proliferation and increased apoptosis as measured by cleaved caspase-3. Migration and invasion were also inhibited by AR knockdown and treatment with Enza was associated with changes in cellular morphology from stellate to round. Additionally, basal-like HCC1806 and mesenchymal stem-like SUM159PT xenografts, with relatively low AR expression, treated with Enza exhibited decreased viability and increased necrosis. Response to Enza in non-LAR xenografts with relatively low AR expression provides promising preclinical data that TNBC patients with relatively low AR expression may also benefit from anti-androgen therapy. Indeed, results of the TBCRC011 trial demonstrate that some non-LAR TNBC benefit from treatment with bicalutamide [54]. This emerging clinical data in support of anti-androgen therapy for the treatment of AR+ TNBC, regardless of molecular subtype, further underscores the possible utility of classifying TNBC as AR+ or quadruple negative breast cancer.

Amphiregulin (AREG), an epidermal growth factor receptor (EGFR) ligand, was identified as an AR-regulated gene and exogenous AREG partially rescues decreased proliferation, migration, and invasion resulting from AR inhibition in vitro [3], suggesting that activation of EGFR is one mechanism by which AR affects TNBC biology. An association between AR and EGFR activity in TNBC was also identified by Cuenca-Lopez et al. [55]. In TNBC tumor lysates, AR protein expression significantly correlated with phosphorylation of EGFR and platelet-derived growth factor receptor beta (PDGFR β). Combined treatment of the AR antagonist bicalutamide with inhibitors of EGFR, PDGFR β , and Erk1/2 exhibited an additive anti-proliferative effect and decreased AR protein expression in non-LAR TNBC cell lines [53].

Graham and collaborators found that cross talk between AR and zinc finger E-box binding homeobox 1 (ZEB1), a transcription factor associated with epithelial-to-mesenchymal transition, influences migration of MDA-MB-231 and MDA-MB-435 cells [56]. ZEB1 can bind the AR promoter and ZEB1 knockdown decreases AR transcript, protein, and downstream AR targets. Likewise, treatment with the AR antagonist bicalutamide decreased ZEB1 protein expression. In the presence of ZEB1 knockdown, bicalutamide decreased migration. By tissue microarray, ZEB1 and AR expression were positively correlated in ER-/PR- BC tissues [56].

Clinical Efficacy of AR Inhibitors in AR+ TNBC

Recent clinical trials evaluating the efficacy of AR antagonists in AR+ TNBC are promising. A phase II trial of bicalutamide in AR+/ER-/PR- metastatic breast cancer found a 6-month clinical benefit rate (CBR) of 19 % [10]. The median progression free survival (PFS) was 12 weeks and bicalutamide was well-tolerated. While bicalutamide permits AR nuclear localization and disrupts the major coactivator binding surface on AR [57], Enza induces a conformational change within AR that in addition to blocking coactivator interactions attenuates the DNA binding of the receptor [58]. A phase II trial of Enza in AR+ TNBC is currently underway and initial results are favorable [14]. Traina et al. report a 16-week CBR of 35 %. The median PFS was 14.7 weeks and Enza was well-tolerated. An androgen-related gene signature was associated with a favorable clinical outcome. While a treatment devoid of chemotherapy side effects is intensely intriguing for patients and their providers, it is important to recognize that clinical investigators select patients with a more indolent clinical course to participate in these clinical trials, thus potentially enriching the patient population for those with more AR-driven tumors. Indeed, 55 % of the patients in this trial had >10 % AR expression.

Although not exclusively enrolling TNBC patients, other AR-directed therapy trials are underway including the CYP17 inhibitor abiraterone (NCT00755885), the androgens DHEA and CR1447 (4-OH-testosterone, NCT0200375, and NCT02067741, respectively), and antisense oligonucleotides targeting AR (NCT02144051). Multiple new generation AR inhibitors are also being tested in clinical trials for prostate cancer including ARN509 (NCT01946204) [59] and ODM-201 (NCT01429064) [60] that inhibit nuclear translocation. Until relatively recently, the discovery of AR antagonists was accomplished using empirical screens to identify molecules that exhibited high affinity receptor binding, the agonist/antagonist efficacy of which was subsequently determined using appropriate cellular/animal models. However, more contemporary, mechanism-based approaches have been developed that leverage the observation that the pharmacology of an AR ligand is determined by its impact on receptor structure and coregulator recruitment [58]. Using this approach, Joseph et al. identified a series of non-competitive AR antagonists which freeze AR in an “apo-conformation” and which attenuate AR action in models of castrate-resistant prostate cancer [61]. This general approach has also yielded AR antagonists which, by virtue of the conformational change they induce in AR, inhibit the activity of all of the clinically relevant AR mutations including F876L. For example, selective androgen receptor degraders (SARDs), compounds that induce proteasome dependent AR degradation and thus remove the receptor as a target for androgens and of pathways that converge on the receptor to enable ligand independent activation, have also been identified (D P McDonnell, JD Norris and J. Katzenellenbogen *pers commun*). An equally exciting new class of AR degraders was recently reported by Gustafson et al. where hydrophobic moieties (degrons) are attached to a high affinity AR ligand. The degron functionality targets the occupied receptor to the proteasome where it is quantitatively degraded [62]. The evaluation of several of these functionally distinct antagonists in breast cancer is currently underway.

Discussion

TNBC is an aggressive BC subtype for which no effective targeted therapies are available. While the exact percentages vary across studies, it is clear that a significant percentage of TNBCs express AR. Nuclear localization of the receptor suggests active AR signaling, and preclinical data indicates that AR+TNBC may critically depend on AR signaling for growth. The existence of the LAR TNBC subtype, with strong AR expression driving a luminal-like expression pattern in the absence of ER, is evidence that AR signaling can play a strong role in the biology of TNBC tumors. AR expression is associated with decreased proliferation in TNBC, but LAR tumors

have a particularly poor prognosis, possibly because of their poor response to chemotherapy. Anti-androgens have shown particular efficacy in preclinical studies of LAR models and may be useful in improving the treatment of LAR tumors. Importantly, response to AR antagonists may not be limited to the LAR subtype, as the results of preclinical studies performed *in vitro* and *in vivo* have demonstrated that some cell lines with relatively low AR expression are sensitive to the newer generation anti-androgen Enza.

Recently reported and ongoing clinical trials using bicalutamide or Enza in TNBC have shown an increase in PFS, suggesting that AR-targeted therapies may improve patient prognosis and supporting a reclassification of TNBC into AR+ and “quadruple negative” disease. To date, there have been many classifications of TNBC subtypes which will likely lead to novel targeted therapeutics. However, we propose prioritizing the classification of AR+ and AR− disease because, unlike the other TNBC subtypes, the therapeutic target is clear, FDA-approved AR-targeted therapies are available, and early clinical trials demonstrate patient benefit from treatment with AR antagonists. Endocrine-targeted therapies such as tamoxifen and aromatase inhibitors have greatly improved the outcomes of ER+ breast cancer, and AR-targeted therapies have improved the prognosis of prostate cancer. Thus, treatment of hormone-dependent cancers has significantly benefited from endocrine-targeted therapy. Although profiling has identified multiple TNBC subtypes, it is reasonable to prioritize classification of AR+ and AR− disease as it is most likely to improve patient outcomes in the near future.

Additional research is needed to identify AR+ TNBC patients who will respond to AR-targeted therapies. Indeed, a signature of genes may more reliably predict responsiveness to anti-androgens than levels of AR alone and such a signature will likely be forthcoming from current trials of Enza in AR+ TNBC. Furthermore, preclinical studies are needed to determine if AR-targeted therapies will be most effective if administered concurrently with chemotherapy, following chemotherapy, or perhaps as a targeted alternative to chemotherapy in AR+ TNBC patients with tumors expressing an “AR-responsive signature.” Further preclinical modeling will also determine whether anti-androgen therapy might synergize with other targeted therapies of current interest such as mTOR, CDK4/6, or EGFR inhibitors. It is also possible that a subset of AR+/HER2 overexpressing TNBC might be sensitive to the combination of an anti-androgen with HER2-directed therapy. Finally, research to determine possible mechanisms of resistance to anti-androgen therapy in TNBC cell lines by methods such as synthetic lethal screening and gene expression profiling of tumor specimens from the Enza trial and others will help to inform future clinical trial design and improve therapy for AR+ TNBC patients. The discovery that a hormone receptor with multiple FDA-approved antagonists may be critical for growth of a subset of TNBCs is an exciting

development. Further preclinical research with AR-targeting drugs as single agents, combined with chemotherapy, or rationally determined targeted therapies, and then ultimately further clinical trials will establish whether an AR-directed agent will represent the first effective targeted therapy for AR+ TNBC.

Conflict of Interest The authors declare that they have no competing interests.

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Androgen Receptor Supports ER α Activity and Genomic Binding in Breast Cancer

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SUMMARY (146/150)

Androgen receptor (AR) is expressed in 90% of estrogen receptor alpha positive (ER+) breast tumors, but its role in tumor growth and progression remains controversial. We demonstrate that AR is required for ER genomic binding and estrogen-mediated proliferation of ER+/AR+ breast cancer. In the presence of estradiol, AR becomes localized to the nucleus in an ER-dependent manner and to binds to chromatin at unique sites containing degenerate estrogen response elements. Inhibition of AR nuclear localization with new generation anti-androgens decreased estradiol-induced ER genomic binding, transcriptional activity, and consequent proliferation, as did AR knockdown. Furthermore, enzalutamide significantly decreased viability of ER+/AR+ patient-derived xenografts and tamoxifen-resistant MCF7 tumors *in vivo*. Our data provide promising pre-clinical evidence that anti-androgens that block AR nuclear localization may be effective in combination with current anti-estrogen therapies such as tamoxifen, fulvestrant, or aromatase inhibitors, and in tumors resistant to traditional breast cancer endocrine therapies.

INTRODUCTION (498/500 words)

AR is even more frequently expressed in breast cancer than estrogen receptor alpha (ER) or progesterone receptor (PR)¹; however the role of AR in breast cancer is complex and dependent on the hormonal milieu, and therefore remains controversial. AR expression is associated with better prognosis in ER+ breast cancer²⁻⁴, possibly due to the fact that like ER, AR positivity is indicative of a more well-differentiated state. In the presence of estradiol (E2), the androgen dihydrotestosterone (DHT) decreases E2-induced proliferation² and ER transcriptional activity⁵ leading to the conclusion that AR is protective in breast cancer. *De novo* or acquired resistance to anti-estrogen therapies is a frequent occurrence, and ultimately all metastatic ER+ breast cancers are resistant^{6,7}, and there is accumulating evidence that androgen signaling and AR may be involved in breast cancer progression and endocrine resistance. In ER+ tumors that respond to neoadjuvant endocrine therapy, AR mRNA and protein expression decrease, while in tumors that fail to respond, AR does not decrease^{8,9}. In laboratory studies, AR over-expression results in resistance to tamoxifen (tam) and aromatase inhibitors (AIs) *in vitro* and *in vivo*^{10,11}. One mechanism of resistance to anti-estrogen therapies may therefore be tumor cell adaptation from estrogen to androgen dependence. Since AIs block conversion of androgens to estrogens, levels of circulating androgens increase in patients on AIs. High levels of the adrenal androgen dehydroepiandrosterone sulfate (DHEA-S) before treatment are predictive of failure on AIs and circulating DHEA-S increased during treatment in patients with tumors that progressed during AI treatment¹². Patients with tumors exhibiting a high ratio of percent cells positive for AR versus ER protein are more likely to have recurrent disease while on tam and also have a worse overall prognosis compared to those with a more equal ratio of these two receptors, as is found in normal breast epithelium¹³. Thus, although AR is associated with a better prognosis, anti-androgen therapies may benefit patients with AR+ breast cancers if the tumors are dependent on AR.

The observation that AR agonists can be anti-proliferative and interfere with ER action does not necessarily indicate that AR antagonists must have the opposite effect. Based on our observations that the new generation AR antagonist enzalutamide (enza) decreases ER-mediated tumor growth, we hypothesized that AR may positively modulate ER activity and that anti-androgens could be utilized either in conjunction with ER-directed therapies or in tumors with *de novo* or acquired resistance. Herein we demonstrate for the first time that AR supports ER genomic binding, that E2 drives nuclear localization of AR, and that AR inhibition significantly decreases E2-induced growth of ER+/AR+ cell line and patient-derived xenografts, as well as tam-resistant tumors *in vivo*. Importantly, these data suggest that patients with ER+/AR+ breast cancer may benefit from combining anti-androgen therapy with anti-estrogen therapy, and that tumors resistant to traditional ER-directed therapies may be responsive to AR-directed drugs, particularly new-generation anti-androgens that inhibit AR nuclear localization or cause AR degradation since AR knockdown gives results similar to inhibition of AR nuclear localization.

RESULTS

AR inhibition impairs E2-induced growth

The role of AR in ER+/AR+ breast cancer remains controversial, with conflicting data suggesting either proliferative or protective effects on breast cancer cell growth *in vitro*^{2,5,14-17}. To determine the role of AR in ER+/AR+ breast cancer we utilized the new-generation anti-androgen enzalutamide (enza), which inhibits nuclear translocation of AR¹³, as well as shRNAs targeting AR in three ER+/AR+ breast cancer cell lines (MCF7, T47D, and ZR-75-1). In media containing full serum, which contains sufficient estrogen to induce ER activity and genomic binding¹⁸, (and androgens to induce AR activity?) enza treatment significantly decreased growth of MCF7 cells (Fig. 1a) as well as T47D and ZR-75-1 cells (Extended Data Fig. 1a) in a concentration-dependent manner. Similar growth inhibition was also observed in. Enza also decreased colony size of MCF7 cells (Fig. 1b) and T47D cells (Extended Data Fig. 1b) in soft agar growth assays conducted using complete culture media, similar to the effect of tam. To confirm this was not due to off-target effects of enza, we silenced AR expression in MCF7 cells using two different shRNA constructs, and AR protein was confirmed to be decreased by western blot (Extended Data Fig. 1c – in progress). AR knockdown led to a significant decrease in MCF7 cell growth over the course of 7 days (Fig. 1c), further demonstrating that AR is required for baseline proliferation of ER+/AR+ breast cancer cells in hormone-replete conditions.

To test the effect of anti-androgens on E2-induced growth, we hormone starved MCF7 cells for 3 days followed by treatment with E2 in the presence or absence of Enza. As expected, 10nM E2 significantly increased growth of MCF7 cells. Even though enza does not bind to ER by ligand binding assay¹³, it decreased E2-induced proliferation in a concentration-dependent manner (Fig. 1d). Similar results showing enza decreased E2-induced growth in a concentration-dependent manner were obtained in T47D and ZR-75-1 cells (Extended Data Fig. 1d) as well as ER+/AR+ PT12 cells recently created from a patient-derived xenograft¹⁹ (Extended Data Fig. 1e). Cell cycle analysis of E2-treated MCF7 and T47D cells demonstrated that enza decreased the percentage of cells in the S and G2/M phases compared to E2 treatment alone (Fig. 1e and Extended Data Fig. 1f). shRNA silencing of AR also significantly decreased estrogen-induced proliferation of MCF7 cells compared to control cells transduced with non-targeting shRNA (Fig. 1f). Together, these data demonstrate that AR inhibition diminishes estrogen-driven proliferation in ER+/AR+ breast cancer cells.

E2 induces AR nuclear translocation

Since we observed that enza and AR knockdown decreased E2-induced growth, we tested whether AR becomes localized to the nucleus following the addition of E2. MCF7 cells were hormone starved for 72 hrs then pre-treated with either vehicle or enza for 3 hrs. Cells were then treated with E2 or DHT plus or minus enza for 3 hrs, and nuclear and cytoplasmic protein fractions were isolated. As expected, DHT induced a strong increase in AR nuclear localization, which was largely blocked by co-treatment with enza (Fig. 1g). However, E2 treatment also increased nuclear AR, and this effect was also blocked by enza (Fig. 1h). E2-induced nuclear localization of AR was also observed in ZR-75-1 (Extended Data Fig. 2a). **Importantly, nuclear translocation of AR in response to E2 was blocked by pretreatment with**

fulvestrant, demonstrating that E2-induced AR nuclear translocation is ER-dependent (Figure XX – still in progress). Furthermore, E2 did not induce AR nuclear localization in ER-/AR+ MDA-MB-453 cell line (Extended Data Fig. 2b). Immunofluorescent staining also showed nuclear localization of AR in response to E2, and this was blocked by enza but not bicalutamide (Extended Data Fig. 2c). This further suggests that the observed AR nuclear localization is not due to promiscuous binding of E2 to AR, but rather that AR translocates to the nucleus in an ER-dependent manner upon E2 stimulation in ER+/AR+ breast cancer cells.

ER and AR co-localize in the nucleus in response to E2

Since both ER and AR moved to the nucleus in response to E2 treatment, we next tested whether they were physically in close proximity. We utilized proximity ligation assay (PLA) technology, which creates a fluorescent signal only when two proteins are in close proximity (within 30-40 nm). MCF7 cells treated with 10nM E2 for 1hr demonstrated a strong increase in PLA signal when probed for ER and AR compared to vehicle control or Enza treatment alone. This E2-induced increase in PLA signal was almost completely inhibited by pre-treatment with Enza (Fig. 1i, j). Similar results were observed in T47D cells (Extended Data Fig. 2c-e).

AR inhibitors diminish ER genome binding

Since AR is a transcription factor and has been previously shown to be capable of interacting with ER and EREs when bound to ligand^{2,5}, we hypothesized that inhibiting AR nuclear localization may alter ER genomic binding. MCF7 cells were hormone starved for 3 days, then pre-treated for 3 hr with vehicle, enza, or MJC13, a second inhibitor of AR nuclear translocation that works by a different mechanism than Enza²⁰. Cells were then treated with E2 for 1hr in the continued presence of vehicle or anti-androgen. Following treatment, global ER ChIP-seq analysis was performed. A minimum of 23 million aligned reads were acquired, and after applying stringent criteria we identified 10,852 ER binding events in E2-treated MCF7 cells (Fig. 2a). Surprisingly, the addition of Enza or MJC13 to the E2-treated cells dramatically decreased ER genomic binding, with the vast majority of ER binding sites displaying an approximately 50% decrease in ER binding in the presence of enza (4,659 sites, 42.9% reduction) or MJC13 (4460 sites, 41.1% reduction)(Fig. 2a-c). ER binding at previously-characterized ER binding sites including *GREB1*, *GATA3*, and *PGR* was tested by qRT-PCR after ChIP, and these experiments confirmed the approximately 50 percent decrease in ER binding intensity in the presence of Enza or MJC13 (Fig. 2d-e). This suggests that the interaction of AR and ER is critical for efficient ER genomic binding in response to E2, and that inhibition of nuclear AR localization decreases E2-induced ER activity by diminishing ER genome binding.

E2 induces a distinct AR DNA binding profile

Since AR is localized to the nucleus in response to E2 and affects ER genome binding, we next assessed AR genome binding in response to E2 to investigate a possible role in ER activity. Hormone-starved MCF7 cells were treated with E2 for 1 hr, or DHT for 4 hrs for comparison, followed by global AR ChIP-seq analysis. As expected, DHT treatment induced a significant increase in AR genome binding compared to vehicle treatment (Fig. 3a-b). We identified 1,813 AR binding events in DHT-treated MCF7 cells. Among these binding sites, 897 (49.4%) were previously identified as being bound by AR in LNCaP, a

prostate cancer cell line, and 1,335 sites (73.6%) were bound by AR in MDA-MB-453, an ER-/AR+ breast cancer cell line²¹ (Extended Data Fig. 3a). These results indicate that DHT-induced AR binding may be more similar between luminal breast cancer cell lines than between breast and prostate cancer cell lines.

In E2-treated MCF7 cells we identified 1,380 AR binding events (Fig. 3a-b). Pre-treatment with Enza abolished E2-induced AR genomic binding, which is consistent with inhibition of AR nuclear localization and previously published reports in prostate cancer²². AR binding could be clearly divided into sites significantly bound by AR only in response to DHT, sites bound only in response to E2, and those bound both in response to DHT and to E2 (Fig. 3a). Only 638 loci were bound by AR in response to both DHT treatment and E2 treatment (25% of all AR bound sites), indicating a large shift in genomic binding sites in response to the different hormones (Fig. 3c). AR binding at previously-characterized AR and ER binding sites was also tested using qRT-PCR after ChIP (Fig. 3d-e). DHT, but not E2, induced a robust increase in AR binding at previously-characterized AR binding sites *FKBP5* and *ZBTB16*. Both E2 and DHT treatments resulted in AR binding to *GREB1* and *GATA3* loci, but only E2 treatment resulted in AR binding at the *PGR* locus (Fig. 3d-e).

The most highly-enriched motif among AR binding sites identified both in response to DHT and in response to E2, as well as sites unique to DHT treatment, was a forkhead motif (Fig. 3a). This result is in agreement with previous studies demonstrating a high degree of overlap between AR and FOXA1 binding sites in breast cancer cells²¹. Interestingly, the most highly-enriched motif among AR binding sites unique to the E2 treatment was a slightly degenerate estrogen response element (ERE) (Fig. 3a). We also analyzed the frequency of consensus AREs and EREs in the different binding site groups. While the frequency of consensus ARE palindromic sites was similar among the three groups of binding sites (Extended Data Fig. 3b), the frequency of AR binding to consensus ERE palindromic sites was dramatically different. These full palindromic EREs comprised only 0.5% of the AR binding sites unique to DHT, but 31.1% of sites unique to E2, and 8.6% of sites bound in response to each ligand (Extended Data Fig 3c). Because of the large differences in ERE prevalence, we compared AR binding sites in response to E2 or DHT stimulation with E2-induced ER binding sites. We found that 75% of AR binding sites following E2 stimulation overlapped with E2-induced ER binding, but only 39% of AR binding sites following DHT stimulation overlapped with E2-induced ER binding (Fig. 3f). In other words, E2 induces AR genomic binding with a strong similarity to E2-induced ER binding.

We then further analyzed the effect of enza and MJC13 on ER binding and found that ER bound sites that overlap AR binding are more strongly impacted by these inhibitors of AR nuclear localization. Using Wilcoxon rank tests, the effect is highly significant for both enza ($p=1.209\text{e-}07$, Extended Data Fig. 3d) and MJC13 ($p=2.498\text{e-}07$, Extended Data Fig. 3e). Taken together, these data demonstrate that in response to E2, AR and ER co-occur at a significant number of loci, and ER binding is most strongly impacted by anti-androgens at these loci.

Enza synergizes with anti-estrogens

Since enza inhibited ER genome binding, we hypothesized that it may act synergistically with anti-estrogens such as tam or fulvestrant in ER+/AR+ breast cancer cells. MCF7 cells were treated with 10nM E2 and varying concentrations of Enza and/or Tam. Four of nine tested combinations of enza plus tam showed synergistic inhibition of E2-induced growth as determined by CalcuSyn²³, while the remaining combinations showed additive effects (Fig. 4a). The combination of enza plus tam also reduced MCF7 growth in soft agar more significantly than either drug alone (Extended Data Fig. 4a). We also tested for synergy between enza and fulvestrant using BCK4 cells. Eight of nine tested combinations showed synergy, while the ninth showed additive effects on E2-induced growth (Fig. 4b). Similar data was obtained in PT12 cells, an ER+/AR+ cell line generated from a patient-derived xenograft, as well as in ZR-751 cells (Extended Data Fig. 4b-c).

Enza inhibits E2-induced tumor growth

Since enza synergized with Tam and fulvestrant in multiple cell lines *in vitro*, we tested the combination of enza plus Tam *in vivo* using an MCF-7 orthotopic xenograft model. GFP-Luciferase labeled MCF-7 cells were injected bilaterally into the number 4 mammary fat pad of nude mice and E2 pellets were implanted subcutaneously on the same day. Once tumors were established, mice were randomized to receive control chow (CTRL), a Tam pellet implanted subcutaneously (Tam), enza-containing rodent chow (Enza), or Enza-containing chow plus Tam pellet (Enza+Tam). Both Enza and Tam significantly decreased tumor viability independently as compared to the control group as measured by luciferase activity (Fig. 4c). Because the decrease in tumor viability by each drug was so strong, the combination of Enza plus Tam did not result in a statistically significant further decrease in viability. The mice receiving enza + tam did show a trend toward longer survival, though this did not reach statistical significance (Fig. 4d).

We also performed microarray expression analysis on 6 tumors from each group. Unsupervised hierarchical clustering showed that enza and tam had largely different effects on gene expression patterns (Extended Data Fig. 5a). This gene expression data was overlayed with AR ChIP-seq data, and we identified 16 genes significantly downregulated in enza-treated tumors that were also bound by AR in response to E2 in MCF7 cells *in vitro*. These genes included *BCL6*, which promotes breast cancer cell survival²⁴, and *NDRG1*, which is associated with shorter disease-free and overall survival in breast cancer²⁵. **Decreased expression of these genes in enza-treated tumors was confirmed by qRT-PCR (Extended Data Fig. 5b – still in progress).**

To confirm that the ability of enza to inhibit tumor growth was not cell-line specific, we also performed a xenograft experiment with PT12 cells, a line recently derived from an ER+/PR+/AR+ patient derived xenograft¹⁹. Following orthotopic injection of GFP-luciferase expressing PT12 cells, mice were implanted with either E2 or DHT pellets and randomized to receive either enza or control chow. Although E2 induced more rapid tumor growth, DHT also stimulated tumor growth. Enza significantly decreased viability of both E2- and DHT-driven tumors in mice (Extended Data Fig. 6a-b), and significantly decreased proliferation in E2-driven tumors (Extended Data Fig. 6c) and increased apoptosis in DHT-driven tumors (Extended Data Fig. 6d).

Enza inhibits Tam-resistant cell growth

Resistance to currently-used endocrine therapies is a common occurrence facing ER+ breast cancer patients. Therefore, we also tested whether Enza could inhibit growth of tamoxifen-resistant MCF7 (MCF7-TamR) cells²⁶. *In vitro*, both enza and MJC13 significantly decreased growth of MCF7-TamR cells (Fig. 5c). Enza also decreased growth of MCF7-TamR cells in soft agar (Fig. 5d), and the combination of enza+tam was more effective than Enza alone.

To test whether enza could inhibit growth of Tam-resistant tumors *in vivo*, 1×10^6 GFP-luciferase expressing MCF7-TamR cells were injected bilaterally in to the mammary fat pads of nude mice. When tumors reached approximately 100 mm^3 , mice were randomized into 4 groups receiving either control chow (CTRL), Enza-containing chow (Enza), slow-release Tam pellets (Tam), or Enza + tam. Tumor viability was monitored by luminescence over time. At 20 days, the Enza-treated mice demonstrated a significant decrease in tumor viability compared to those in the CTRL group (Fig. 5e-5f). Each treatment resulted in a significant decrease in tumor weight compared to CTRL-treated tumors, with enza+tam mice having the smallest tumors at the end of the experiment (Fig. 5f). The combination also resulted in a significant decrease in ER expression compared to CTRL or either drug alone (Extended Data Fig. 6e-f).

AR is expressed in recurrences.

To validate the clinical utility of anti-androgens as a potential therapy for advanced ER+ tumors, we examined primary tumors and local recurrences or metastases from a group of breast cancer patients with clinical outcome data available. Sections of FFPE breast tumors were collected from a cohort of 192 female patients diagnosed with breast cancer at the Massachusetts General Hospital (Partners) between 1977 and 1993, treated with adjuvant tamoxifen and followed at the hospital through 1998. The women ranged in age from 20 to 91 years at the time of cancer diagnosis with a median age of 68 years. Of 49 patients with ER+/AR+ primary tumors that developed local recurrence, 96% of these recurrences were AR+. Further, in 63% of these cases, the ratio of AR to ER expression (% cells positive) was higher in the recurrence compared to the primary tumor. Of 55 patients that developed distant metastasis, 67% of the metastases retained AR expression. In 67% of these cases, the relative expression of AR to ER was higher in the metastasis compared to the primary. This is consistent with other studies demonstrating that AR is more highly expressed in metastases than other hormone receptors including ER and PR²⁷. This suggests that anti-androgens such as enza may be a therapeutic option even for patients with advanced or metastatic disease.

DISCUSSION

AR has long been thought to antagonize ER activity, however our data demonstrate for the first time that AR supports ER genome binding and activity in breast cancer, and that the interplay between these hormone receptors is even more complex than previously understood. In response to E2, AR translocates to the nucleus in an ER-dependent manner and binds chromatin at sites that overlap ER binding sites and are enriched for ERE half sites. Inhibiting nuclear localization of AR with new generation anti-androgens dramatically decreased ER chromatin binding, with the greatest effects observed on sites also bound by AR. The importance of this interaction is demonstrated by the ability of enza, an AR inhibitor, or AR knockdown to decrease baseline and E2-induced growth *in vitro*, and to decrease ER+/AR+ breast tumor growth *in vivo*. This is surprising given previous studies showing that androgens diminish ER activity. However, these observations are not mutually exclusive. Ligand-bound AR may interfere with E2-induced ER activation, likely due to competition between AR and ER for the same genome binding sites; however, in this same study, wild type AR without ligand did not significantly diminish ER activity². We propose that unliganded AR interacts with ER in an E2/ER-dependent manner to support ER genome binding and proliferative activity. Thus, AR inhibitors that prevent nuclear translocation can have the same effect as ligand-bound AR (suppression of ER activity) via a different molecular mechanism. Importantly, the use of AR inhibitors would avoid the androgenic side effects of androgen therapy. This is especially timely given that several clinical trials testing the efficacy of enza in breast cancer are currently underway, including a randomized study testing enza in combination with the AI exemestane in patients with advanced ER+ disease (NCT02007512) and the combination of Enza and Fulvestrant (NCT01597193).

Importantly, our assays were performed using only endogenous AR and ER in cells that naturally express both receptors. In light of recent data from our lab and others suggesting that the ratio of AR:ER protein expression is a predictor of endocrine therapy response¹³ and DCIS progression²⁸, it is likely that the interaction of these proteins may depend on their relative expression, hormone levels in the periphery and in the tumor microenvironment, and/or expression of shared co-factors such as FOXA1. Our data show that across multiple cell lines and preclinical models of ER+/AR+ breast cancer, AR antagonists such as enza and MJC13 that inhibit AR nuclear translocation also indirectly inhibit ER. Thus, we propose that such anti-androgens may be an effective therapy for patients with ER+/AR+ disease. We further show that enza effectively inhibits growth of Tam-resistant tumors in a preclinical model, and that recurrences resulting from endocrine resistance often retain AR positivity. Collectively, our data demonstrate that AR is not solely an inhibitory competitor of ER, but that AR supports ER activity in breast cancer, opening the door to new methods of treating ER+/AR+ breast cancers either in combination with traditional ER-directed therapies or upon resistance to such therapies.

FIGURE LEGENDS

Figure 1 – AR inhibition decreases ER+/AR+ breast cancer growth and AR/ER co-localization. (A) MCF7 cells were cultured in increasing concentrations of enza. (B) MCF7 cells were grown in soft agar in the indicated concentration of enza or tam and colony size was measured by ImageJ. (C) MCF7 cells expressing a non-targeting (shNeg) or AR-targeting (shAR15 and shAR17) shRNA constructs were cultured in supplemented media. (D) MCF7 cells were grown in media with charcoal-stripped serum (CSS) for 72 hrs then treated with vehicle (Veh), 10nM estradiol (E2), or E2 + the anti-androgen Enza or MJC13 and cell number was measured by crystal violet. (E) MCF7 cells were grown in media with CSS for 72hrs then treated with Veh, E2, or E2+enza for 24hrs followed by fixation and cell cycle analysis. (F) MCF7 cells expressing shNeg, shAR15, or shAR17 were cultured in media with CSS for 72 hrs then treated with veh or E2 and growth was measured by crystal violet. (G,H) MCF7 cells were grown in media with CSS for 72hrs then treated with the indicated treatment for 3 hrs, and nuclear extracts were subjected to immunoblotting for AR and TOPOI. (I) MCF7 cells were grown in media with CSS for 72hrs then treated with E2 +/- Enza for 1 hr followed by fixation and PLA staining for AR and ER (red). Nuclei were stained with DAPI (blue). (J) Fluorescent intensity per nuclei was measured by IMAGE ANALYZER(?).

Figure 2 – AR inhibitors diminish ER genomic binding. ChIP-seq for estrogen receptor alpha (ER) in MCF7 cells grown in charcoal stripped serum for 3 days then treated with estradiol (E2) +/- the AR inhibitors enza or MJC-13. (A) Heat map of binding following 1 h of E2 treatment. The heat map is shown with a horizontal window of +/- 2kb. (B) The number of binding sites identified by MACS2, using vehicle treatment as the control, is reduced in cells pre-treated with AR inhibition. (C) After AR inhibition, the ER ChIP-seq signal is lower at each individual binding site and is highly correlated between E2 induction and E2 induction following enzalutimide (blue) or MJC13 (red). (D-E) ChIP-qPCR (D) and ChIP-seq read depth (E) results both indicate reduced ER binding at well-characterized ER binding sites with AR inhibition.

Figure 3 – Estrogen induces AR genome binding that overlaps with ER binding. ChIP-seq for Androgen Receptor (AR) in MCF7 cells grown in charcoal stripped serum for 3 days then treated with estrogen (E2) for 1 h or DHT for 4 h. (A) Heat map of binding following E2 or DHT treatment. The heat map is shown with a horizontal window of +/- 2kb as well as enriched motifs for each category. (B) The number of binding sites identified by MACS2, using vehicle treatment as the control. (C) The number of AR binding sites that are unique to DHT (red), unique to E2 (blue) or shared (overlap) are shown. (D-E) ChIP-qPCR (D) and ChIP-seq read depth (E) results both show AR binding at well-characterized ER binding sites following E2 treatment. (F) The percentage of AR binding sites in response to DHT (left) or E2 (right) that were also identified as ER binding sites (blue) is shown.

Figure 4 – Enza synergizes with tam and fulvestrant *in vitro* and combines with tam *in vitro*. MCF7 cells (A) or BCK4 cells (B) were grown in media with CSS for 3 days followed by treatment with 10nM E2 and the indicated concentration of Enza and/or tam (A) or fulvestrant (B). Percent inhibition was compared to E2+vehicle conditions, and synergy was calculated using Calcsyn software. A Combination Index value less than 1 is indicative of synergistic activity. (C-E) GFP-luciferase expressing MCF7 cells were implanted into the mammary glands of nude mice with estrogen pellets and were randomized into groups to receive either control chow (CTRL), tamoxifen pellets (tam), enzalutamide-containing chow (enza), or tam pellets plus enza chow (tam+enza). (C) Tumor growth was measured over time by luminescence. (D) Survival of mice in each group is shown over time. (E) Representative luminescent imaging of mice from DAY ???.

Figure 5 – Enza decreases ER nuclear localization *in vivo* and inhibits tamoxifen-resistant tumor growth. (A-B) MCF7-TGL cells were implanted into the mammary glands of nude mice with estrogen pellets and randomized into groups to receive either control chow (CTRL), enzalutamide-containing chow (enza), or control chow plus tamoxifen pellets (tam). After XXXX days, tumors were collected and formalin fixed followed by IHC for ER. (A) Percent positivity and staining intensity for nuclear ER are shown. (B) Representative images showing decreased nuclear localization of ER in enza-treated tumors compared to veh- and tam-treated tumors. (C) MCF7-TamR cells were grown in XXX media in the presence of vehicle, tam, enza, or MJC13 and cell number was assessed after 7 days. (D) MCF7-TamR cells were plated in soft agar and the number of colonies was counted after 14 days. (E-F) MCF7-TamR cells were implanted into the mammary glands of nude mice with estrogen pellets and were randomized into groups to receive either control chow (CTRL), tamoxifen pellets (tam), enzalutamide-containing chow (enza), or tam pellets plus enza chow (tam+enza). (E) Tumor growth was measured over time by luminescence. (F) Luminescent images of CTRL or Enza-treated mice on Day 20 (upper). Final tumor weights of mice from each group (lower). (G) Example of AR and ER staining on primary and metastatic tumor tissue demonstrating AR is strongly expressed in metastatic tissue.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1 – Enza and AR knockdown decrease baseline and E2-induced proliferation of breast cancer cells in vitro. (A) ER+/AR+ T47D or ZR-751 cells were grown in complete media with the indicated concentration of enza and cell number was monitored by Incucyte live cell imaging. Growth is expressed as fold change compared to t=0. (B) T47D cells were grown in soft agar in the indicated concentration of enza or tam and colony size was measured by ImageJ. (C) Whole cell extract from MCF7 cells transduced with a non-targeting control (shNeg) or shRNA targeting AR (shAR15 or shAR17) were subjected to western blotting for AR, ER, and tubulin. (D-E) T47D, ZR-751, or PT12 cells were grown in media with CSS for 72hrs then treated with Veh, E2, or E2+enza at the indicated concentrations and cell number was monitored by Incucyte live cell imaging. Growth is expressed as fold change at XXX hrs compared to t=0. (F) T47D cells were grown in media with CSS for 72hrs then treated with Veh, E2, or E2+enza for 24hrs followed by fixation and cell cycle analysis.

Supplemental Figure 2 – E2 induces and enza inhibits AR nuclear localization. (A) ER+/AR+ ZR-751 or (B) ER-/AR+ MDA-453 cells were grown in media with CSS for 72hrs, then pre-treated for 3 hr with enza or vehicle control. Following pre-treatment, cells were treated with veh, 10nM DHT, or 10nM E2 +/- enza as shown for 3 additional hrs. Nuclear extracts were then obtained and subjected to western blotting for AR and TopoI. (C) MCF7 cells were grown in media with CSS for 72 hr then pre-treated with veh, enza, or bicalutamide (bic). Following pre-treatment, cells were treated with veh or 10nM E2 +/- enza or bic as shown for an additional 3 hr. Cells were then fixed and ICC was performed for AR (green) and ER (blue), and nuclei were stained with DAPI (blue). (D) MCF7 cells were grown in media with CSS for 72hrs then treated with veh or E2 +/- enza for 1 hr followed by fixation and PLA staining for AR and ER. (E) The number of fluorescent foci per nucleus was determined by Image Analyzer, and (F) shows the number of nuclei with greater than 20 foci (per 100 nuclei measured).

Supplemental Figure 3 – AR and ER binding in MCF7 cells. (A) Venn diagram illustrating overlap between DHT-induced AR binding in MDA-453 cells (light blue), LNCAP prostate cancer cells (yellow) and MCF7 cells (pink). (B) Percentage of AR binding sites in response to DHT, E2, or either ligand that match the consensus palindromic ARE sequence. (C) Percentage of AR binding sites in response to DHT, E2, or either ligand that match the consensus palindromic ERE sequence. (D-E) Scatter plot of ER ChIP-seq signal intensity with E2 alone (X-axis) versus E2+Enza (D) or E2+MJC13 (E) on the Y-axis. Blue points are ER binding loci that were also bound by AR in response to E2, while red points represents loci not bound by AR in response to E2.

Supplemental Figure 4 – Enza synergizes with anti-estrogens. (A) T47D cells were grown in soft agar in the indicated concentration of enza and/or tam and the number of colonies was quantified using ImageJ. (B-C) ER+/AR+ PT12 cells, a recently isolated cell line from a patient-derived xenograft (B), or

ZR-751 cells (C) were cultured in media with CSS for 72 hr, then treated with 10nM E2 +/- the indicated concentrations of enza and or ICI. (D) T47D cells were grown in complete media supplemented with 10% FBS and treated with the indicated concentrations of enza and/or tam. Proliferation was assessed by Incucyte live cell imaging, and percent inhibition compared to vehicle control was calculated for each treatment. Combination Index (CI) was calculated by Calcosyn software. CI < 1 is indicative of synergistic inhibitory activity.

Supplemental Figure 5 – Enza alters MCF7 xenograft gene expression differently than tamoxifen. GFP-luciferase expressing MCF7 cells were implanted into the mammary glands of nude mice with estrogen pellets and were randomized into groups to receive either control chow (CTRL), tamoxifen pellets (tam), or enzalutamide-containing chow (enza). 11 days after initiation of treatment tumors were excised and flash frozen, and mRNA was isolated. Gene expression profiling was performed on 6 samples from each group, and unsupervised hierarchical clustering was performed.

Supplemental Figure 6 – Enza decreases E2- and DHT-induced growth of a patient-derived xenograft cell line and is effective against tam-resistant tumors. (A-B) 1×10^6 GFP-luciferase expressing PT12 cells were injected orthotopically into the mammary fat pad of NOD-SCID mice and received either an E2 or DHT pellet. When tumors reached an averaged of 100mm³, mice were randomized into the following groups: E2 with control chow (n=10) or enza chow (n=10) or DHT with control chow (n=5) or enza chow (n=5). Tumor viability was measured by IVIS. (C) IHC for BrdU as quantified by ImageJ in tumors from mice with E2 pellets with control or enza chow. (D) IHC for cleaved caspase 3 as quantified by ImageJ in tumors from mice with DHT pellets with control or enza chow. (E) Average weight of excised tumors from MCF7-TamR xenografts. (F) Quantification and (G) representative images of IHC for ER in MCF7-TamR xenograft tumors.

Methods

Cell culture. All cell lines were authenticated by short tandem repeat analysis and tested negative for mycoplasma in January 2015. The BCK4 line is an ER+/AR+ breast cancer line recently derived from a pleural effusion²⁹. For the BCK4 cell line, the patient sample was acquired under a University of Colorado Institutional Review Board approved tissue acquisition protocol and patient-informed consent was obtained to acquire blood and tissue for research purposes. MCF7-TamR cells were obtained from Dr. Doug Yee at the University of Minnesota, and were generated by chronic treatment of MCF7 cells with tam. All other cell lines were obtained from the ATCC. BCK4 and MCF7 cells were grown in MEM with 5% FBS, NEAA, and insulin. MCF7-TamR cells were grown in XXXX. ZR-75-1 cells were grown in RPMI with 5% FBS, T47D cells were grown in DMEM with 10% FBS. All cells were grown in a 37°C incubator with 5% CO₂. MCF7-TGL cells were generated by stable infection with pLNCX2-GFP-Luc vector, encoding a GFP and luciferase and sorted for GFP.

MCF7 AR knockdown cells were generated by lentiviral transduction of shRNAs targeting AR (pMISSION VSV-G, Sigma Aldrich; St Louis, MO), including AR shRNA 3715 (shAR15) and AR shRNA 3717 (shAR17). Lentiviral transduction of pMISSION shRNA NEG (shNEG) was used as a non-targeting control. Plasmids were purchased from the University of Colorado Functional Genomics Core Facility.

Tumor studies. MCF7 experiments with enzalutamide delivered in rodent chow were performed at the University of Colorado Anschutz Medical Campus and approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC protocol 83611(03)1E). All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. For MCF7 xenograft experiments, 10⁶ MCF7-GFP-Luc cells which stably express GFP and luciferase for IVIS imaging purposes were mixed with Matrigel (BD Biosciences) and injected bilaterally into the fourth inguinal mammary fat pad of female, ovariectomized athymic nu/nu mice (Taconic). At time of tumor injection, E2 pellets (60-day release, 1.5 mg/pellet, Innovative Research of America) or the non-aromatizable 5-alpha-dihydrotestosterone (DHT) (8 mg/pellet, packed and sealed in silastic tubing) were implanted subcutaneously (SQ) at the back of the neck. Tumor burden was assessed using *in vivo* imaging system (IVIS) or caliper measurements. Once the tumors established, mice were matched into groups based on total tumor burden as measured by IVIS or caliper. Groups receiving tamoxifen had a 90-day release, 5 mg/pellet (Innovative Research of America) implanted SQ. Mice were administered enzalutamide in their chow (approximately a 50 mg/kg daily dose). Enzalutamide was mixed with ground mouse chow (Cat # AIN-76, Research Diets Inc; New Brunswick, NJ) at 0.43 mg per gram of chow. The feed was irradiated and stored at 4C before use. Mice in the control group received the same ground mouse chow without enzalutamide. All mice were given free access to enzalutamide formulated chow or control chow during the entire study period and at an average of 3.5 g/day food intake. Feed was changed in the animal cages twice a week. Water and feed were prepared ad libitum. Two hours prior to sacrifice, mice were injected IP with 50 mg/kg BrdU (Sigma-Aldrich). Mice were euthanized by CO₂ asphyxiation followed by cervical dislocation and blood, tumors, colon, uteri and mammary glands were harvested.

For the PT-12 tumor study, 6×10^6 cells were injected into the fourth inguinal mammary fat pad of NOD-SCID-IL2Rgc^{-/-} female mice into which a DHT pellet (1.5 mg 60-day release, Innovative Research of America) was implanted SQ. Tumor size was measured using calipers and when tumors reached 100 mm³, the mice began receiving 10 mg/kg enzalutamide or vehicle by oral gavage. Once the tumors reached 400 mm³, another group was started on 25 mg/kg enzalutamide. At the end of the experiment, tumors were weighed and processed for embedding.

Immunohistochemistry. Slides were deparaffinized in a series of xylenes and ethanols and antigens were heat retrieved in either 10mM citrate buffer pH 6.0 (BrdU, Ki67) or 10mM Tris/1mM EDTA buffer at pH 9.0 (AR, ER, caspase 3). Tissue for BrdU was incubated in 2N HCl followed by 0.1M sodium borate following antigen retrieval. Antibodies used were: AR clone 441, and ER clone 1D5 (Dakocytomation), cleaved caspase 3 (Cell Signaling Technology), Ki67 (Santa Cruz sc-15402) and BrdU (BD Biosciences). Envision-HRP (Dakocytomation) was used for antibody detection. TUNEL staining for apoptosis was performed using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore), as per manufacturer's instructions. AR and ER staining was assessed by a pathologist (P.J. or A.T.) and the score is reported as intensity multiplied by percent positive cells or in the case of the tamoxifen treated cohort, the KM curve is based on percent cells positive, although results are similar and still significant when the intensity is multiplied by percent positive. For BrdU and TUNEL staining in xenograft studies, three separate 200X fields of each xenograft tumor were taken using an Olympus BX40 microscope (Center Valley, PA) with a SPOT Insight Mosaic 4.2 camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI). A color threshold (RGB for positive staining nuclei, and HSB for total nuclei) was adjusted manually using ImageJ (National Institutes of Health) for each image, and particles created by the thresholds were analyzed for total area. RGB area was divided by HSB area and multiplied by 100 for each image. For analysis of the nuclear androgen receptor, cleaved caspase 3 and Ki67, slides were scanned at 20x on an Aperio Scan Scope XT. Mammary tumor tissue was traced separately for each tumor and necrotic areas of the tumor removed using a negative pen tool in Aperio's Scanscope software. A Nuclear Algorithm was utilized to measure the percent positive cells for the Ki-67 and Androgen Receptor stained slides and the data exported. Cleaved Caspase 3 stained slides were analyzed using a modified Positive Pixel Count algorithm.

Immunoblotting. Whole cell protein extracts (50 µg) were denatured, separated on SDS PAGE gels and transferred to PVDF membranes. After blocking in 3% BSA in TBS-T, membranes were probed overnight at 4°C. Primary antibodies utilized include: ERalpha (Neomarkers Ab-16, 1:500 dilution), AR (Millipore PG-21, 1:500 dilution), Topo 1 (Santa Cruz C-21, 1:100 dilution) and alpha-tubulin (clone B-5-1-2 from Sigma, 1:30,000 dilution). After incubation with appropriate secondary antibody, results were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer).

Nuclear cytoplasmic fractionation. 1×10^5 cells were seeded in 10cm dishes in medium supplemented with 5% CSS. After three days the cells were pre-treated with 10 µM Enza for 3 hr and then co-treated with either 10 nM DHT for 3 hr in continued presence of Enza or 10 nM E2 for 1 hr in continued presence of Enza. The cells were washed with phosphate buffered saline (PBS) and cellular fractionation

was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Life Technologies) as per manufacturer's instructions.

Proximity ligation assay. 1×10^5 cells per well were seeded into 8-well chamber slides (MANUFACTURER) in medium supplemented with 5% CSS. After 72 hrs, cells were pre-treated with 10 μ M Enza for 3 hr and then co-treated with 10 nM E2 for 1 hr in continued presence of Enza. Cells were washed with PBS then fixed with 4% formaldehyde for 30 min at room temperature and permeabilized with 0.2% triton X-100. Samples were then blocked with 5% BSA for 1 h and incubated with an antibody against AR (AR [N20] Santa Cruz sc-815 1:100) in PBS 0.1% triton overnight. The incubation with the secondary antibody anti-rabbit Alexa Fluor 488 (1:1000) was done in 2.5% BSA for 2 h at ambient temperature. The nuclei were stained with DAPI (1 μ g/ml) for 30 min. Cells were visualized with a 60 X objective and a Qimaging digital camera coupled to an Olympus X71 fluorescence microscope using a yellow fluorescent protein (YFP) filter (Chroma U-N31040). The nuclear distribution of AR (ratio of nuclear AR signal/total AR signal) was quantified in a minimum of 48 cells using ImageJ software.

Real-Time Quantitative Polymerase Chain Reaction (qPCR). cDNA was synthesized from 1 μ g of total RNA, using M-Mulv reverse transcriptase enzyme (Promega). For FASN, PRLR and GCDFP-15, SYBR green quantitative gene expression analysis was performed using the following primers: FASN F 5'-AAGGACCTGTCTAGGTTTGATGC-3', FASN R 5'-TGGCTTCATAGGTGACTTCCA-3'; PRLR F 5'-TATTCACCTGACTTACCACAGGGA-3', PRLR R 5'-CCCATCTGGTTAGTGGCATTGA-3'; GCDFP-15 F 5'-TCCCAAGTCAGTACGTCCAAA-3', GCDFP-15 R 5'-CTGTTGGTGTAAGTCCAG-3'; 18S F 5'-TTGACGGAAGGGCACCACCAG-3', 18S R 5'-GCACCACCACCCACGGAATCG-3'. For PR and SDF-1, taqman real time PCR was performed using validated primer/probe sets from Applied Biosystems (assay IDs: PR Hs01556702_m1, SDF-1 Hs00171022_m1, 18S Hs99999901_s1). Relative gene expression calculated using the comparative Ct method and values were normalized to 18S.

ChIP-seq. 1×10^5 cells were seeded in 15cm dishes in medium supplemented with 5% CSS. After three days the cells were pre-treated with 10 μ M Enza for 3 hr and then co-treated with either 10 nM DHT for 4 hr in continued presence of Enza or 10 nM E2 for 1 hr in continued presence of Enza. The cells were washed with phosphate buffered saline (PBS) then fixed as per manufacturer's instructions (Active Motif). Peak calls were made by MACS2³⁰ with default parameters using the sequence alignments obtained from Active Motif. Motif discovery was performed on 100 base pairs surrounding the peak summit using BioProspector³¹. Patser³² was used to determine significant matches to AREs and EREs.

Cell Cycle. 1×10^5 cells were seeded in 15cm dishes in medium supplemented with 5% CSS. After three days the cells were pre-treated with 10 μ M Enza for 3 hr and then co-treated with either 10 nM E2 for 1 hr in continued presence of Enza. The cells were washed with phosphate buffered saline (PBS) then incubated with Krishan's stain overnight.

AR knockdown.

Cellular Assays and Reagents. Cells were treated with 10 μ M enzalutamide (ENZ, Medivation; San Francisco, CA), 10 nM dihydrotestosterone (DHT, Sigma Aldrich). 10 μ M ENZ approximates the IC₅₀ of the four cell lines studied (data not shown) and is a clinically achievable, well-tolerated treatment concentration (NCT01889238). Androgen concentrations have been previously examined in breast cancer³³ and intratumoral DHT concentrations (249 pg/g) were significantly higher than in blood. The DHT concentration of the present study is consistent with other *in vitro* studies of DHT in breast cancer^{34,35}, and approximates levels of circulating testosterone in obese, postmenopausal women³⁶ as well as DHT levels in fetal bovine serum used during routine tissue culture propagation .

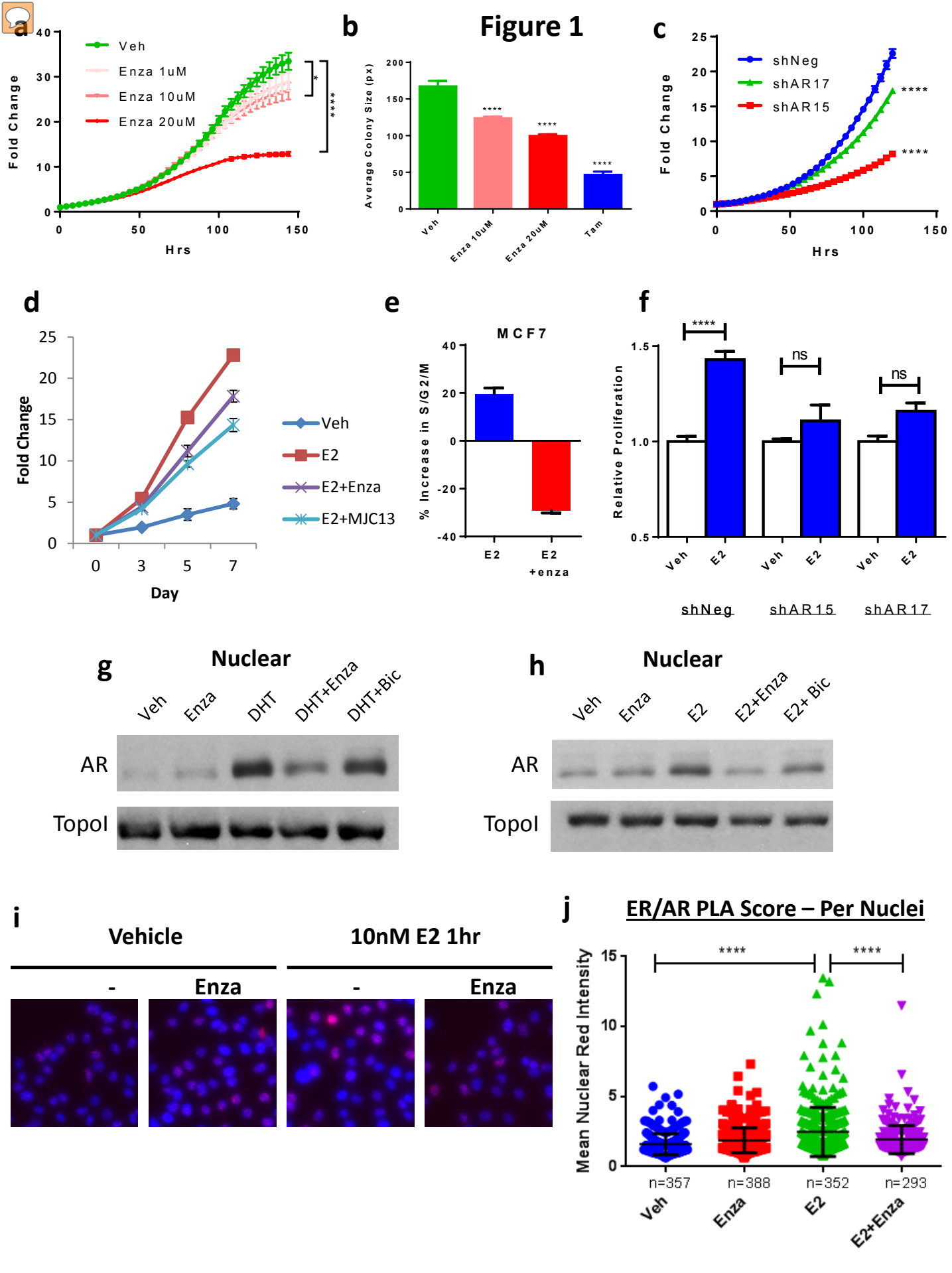
For crystal violet assays, cells were fixed in 10% formalin, rinsed in PBS, and stained with 5% crystal violet. Crystal violet was then dissolved in 10% acetic acid and measured at 540 nm. ☐ MTS assays were performed with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega; Madison, WI) according to the manufacturer's protocol. Proliferation assays were also performed using the Incucyte ZOOM imaging system (Essen BioSciences). Soft agar assays were performed in 0.5% bottom and 0.25% top layer agar (Difco Agar Noble, BD Biosciences).

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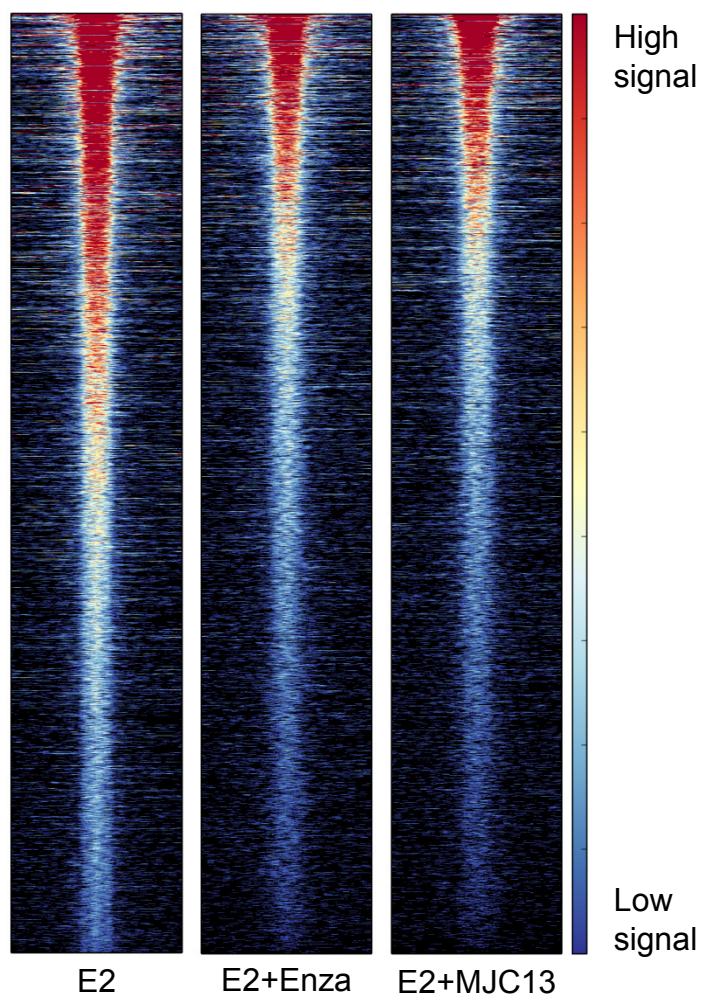
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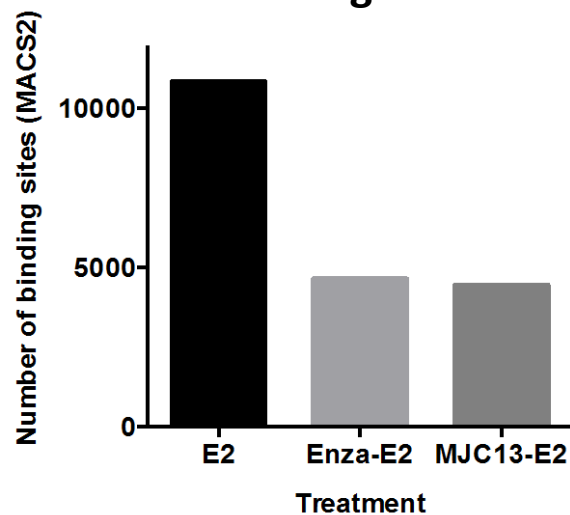
A ER ChIP-seq

ER bound sites

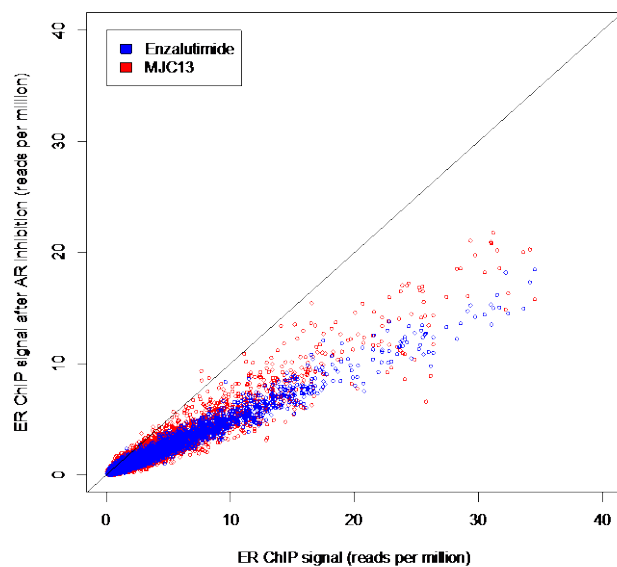


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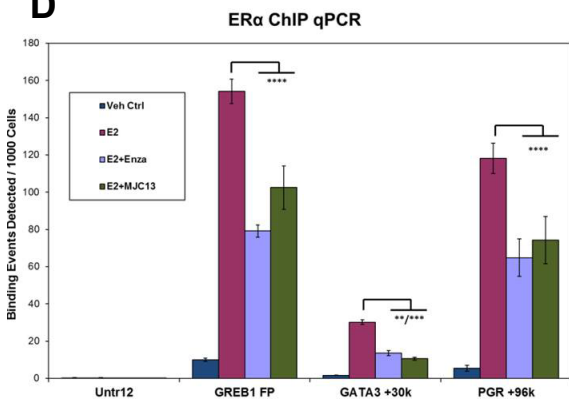
Figure 2



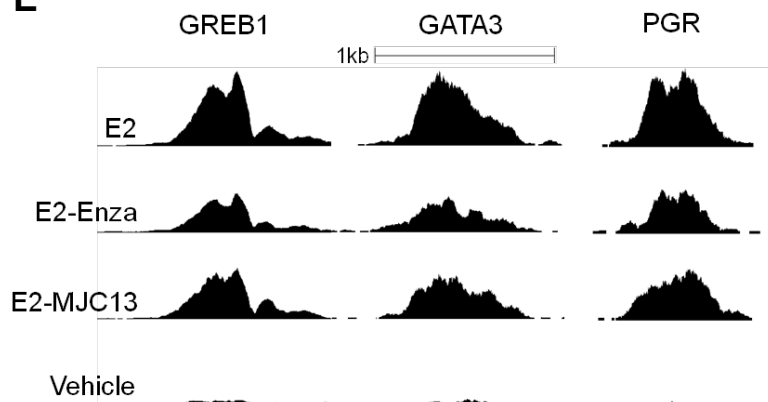
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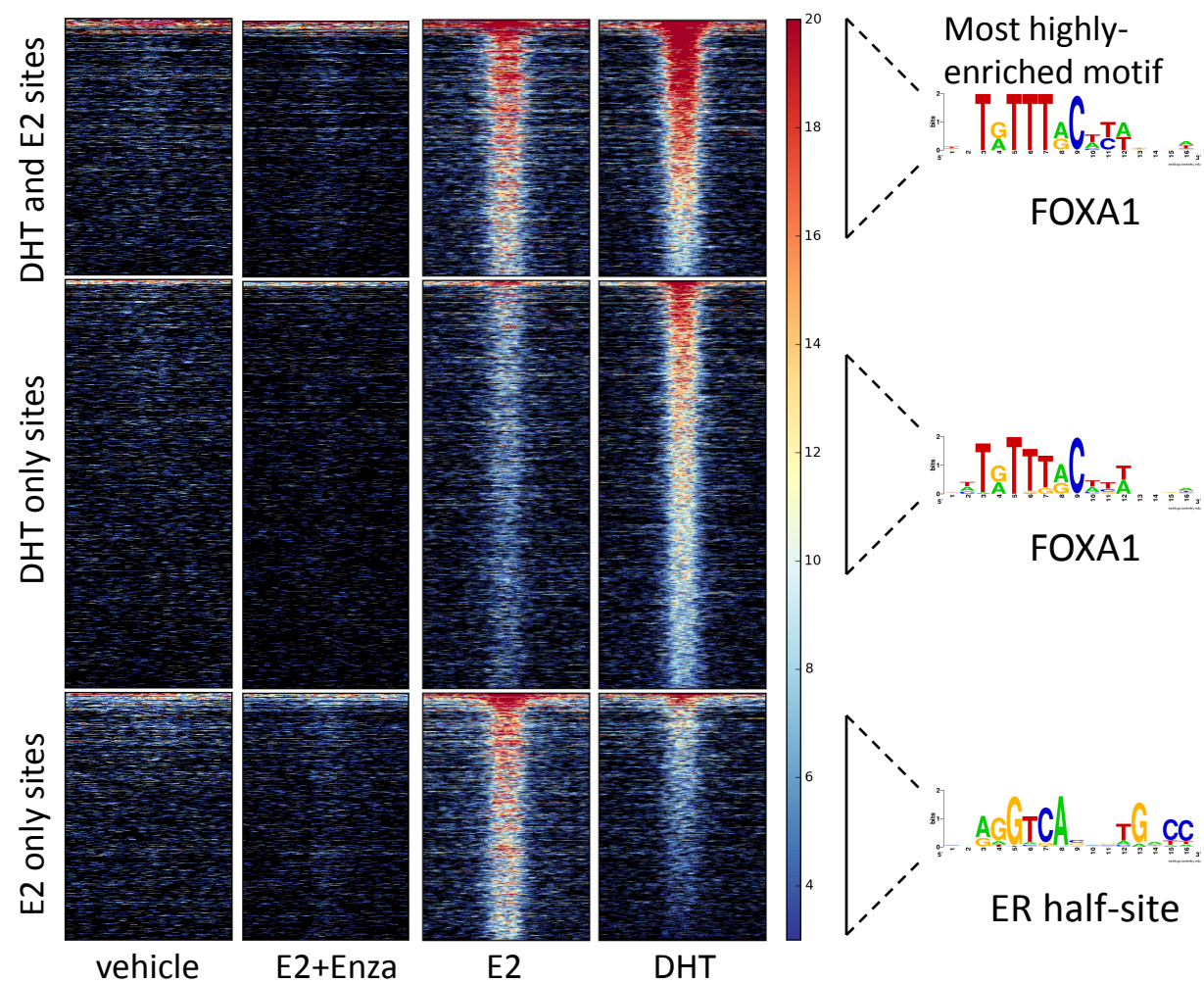
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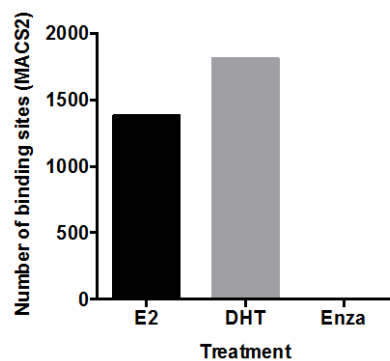


AR ChIP-seq

Figure 3

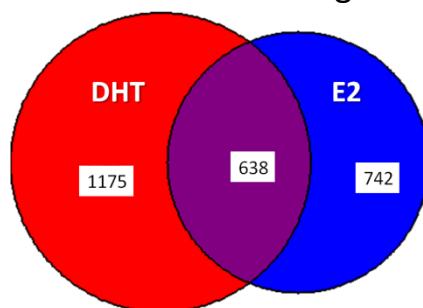


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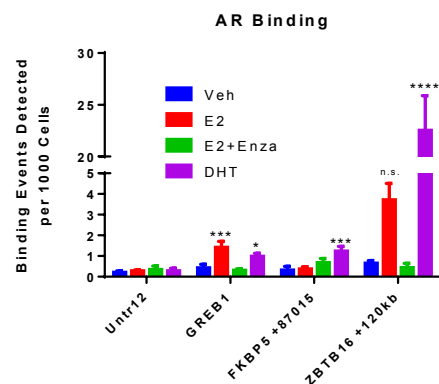


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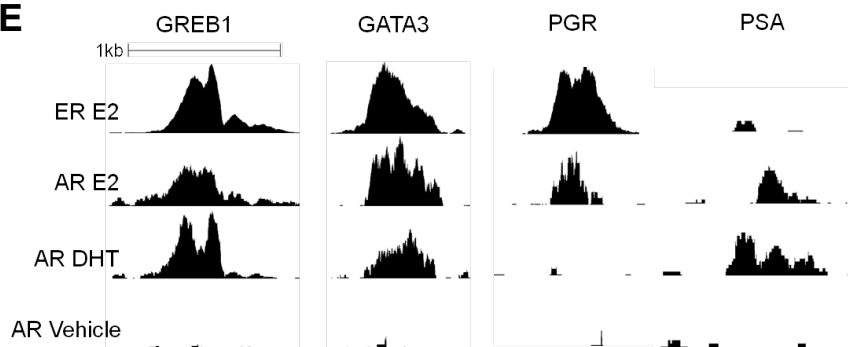
Number of AR binding sites



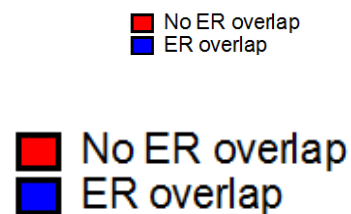
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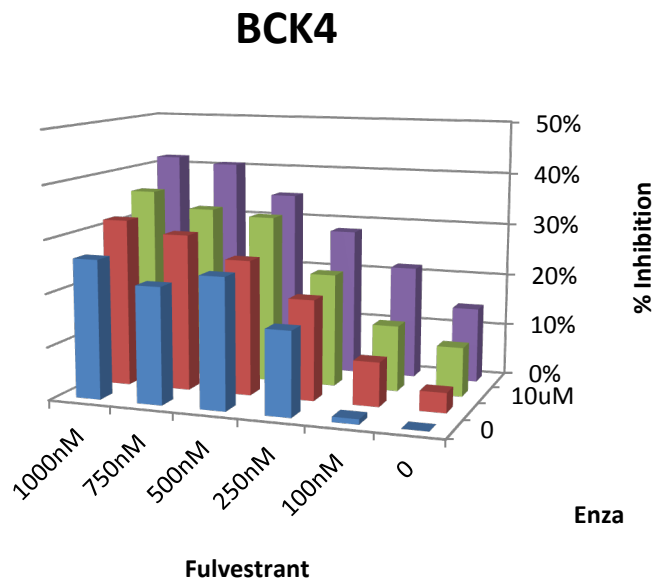
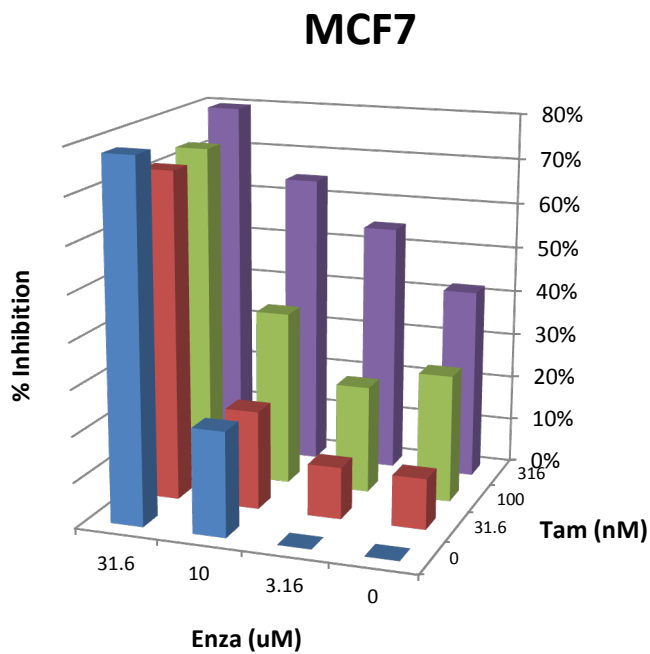


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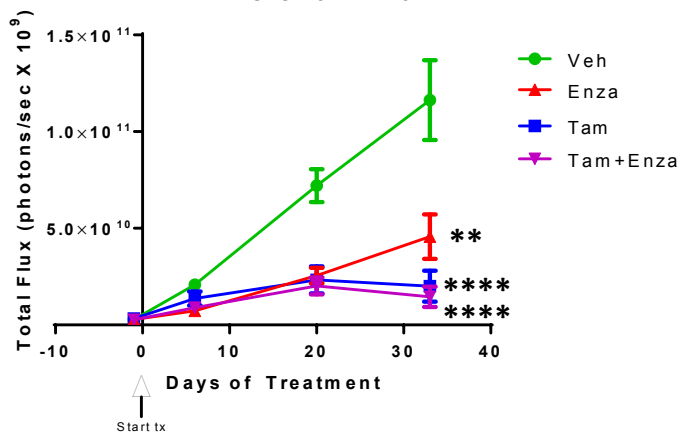
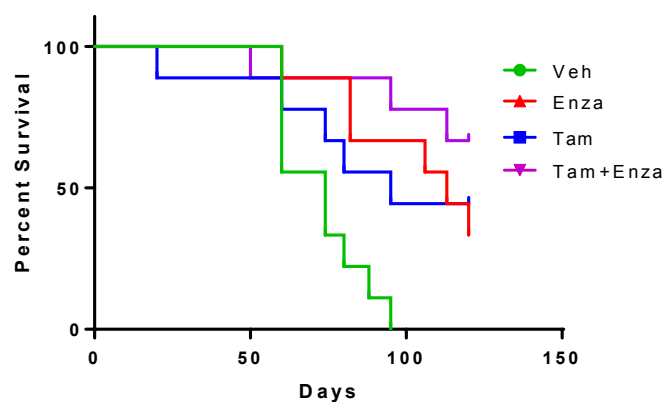
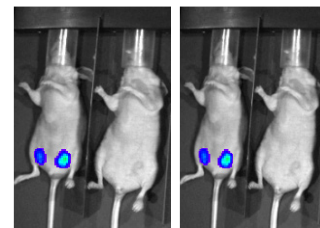
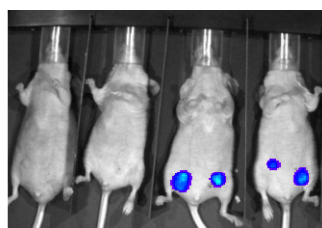
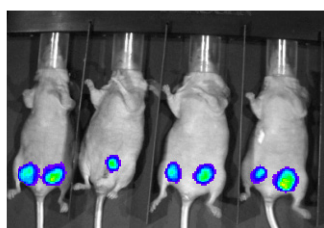
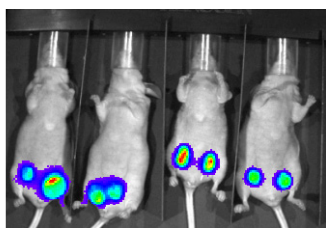


**B****Figure 4****Combination Index**

Enza (uM)	Tam (nM)		
	.01	.1	1
1	1.286	1.261	0.722
10	0.982	0.956	0.791
20	1.396	1.420	1.420

Combination Index

Enza	Fulvestrant				
	100nM	250nM	500nM	750nM	1000nM
5uM	0.831	0.652	0.838	1.027	1.238
10uM	0.882	0.745	0.768	1.015	1.171
20uM	0.835	0.8	0.876	0.938	1.119

C**MCF7 IVIS Over Time****D****MCF7 Survival Curve****E****Veh****Enza****Tam****Tam+Enza**

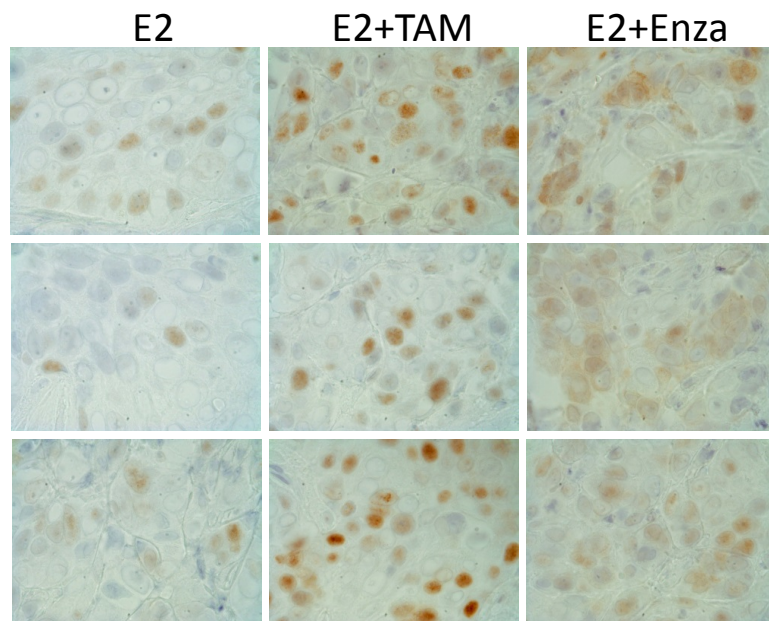
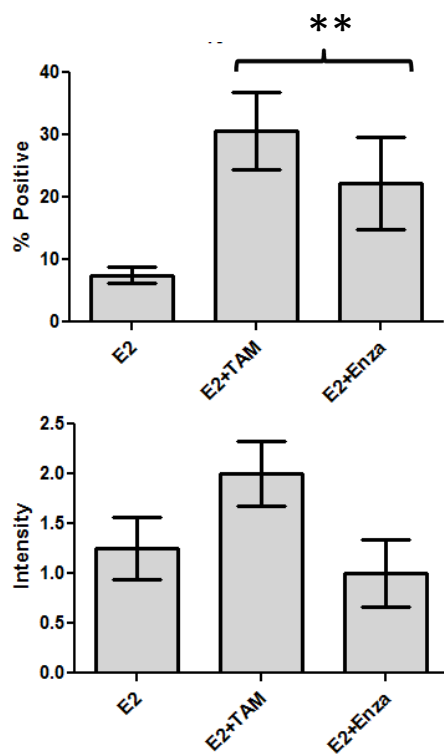
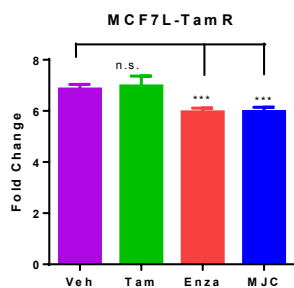
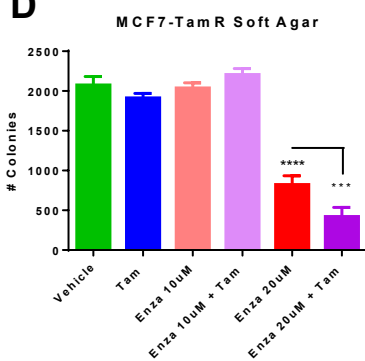
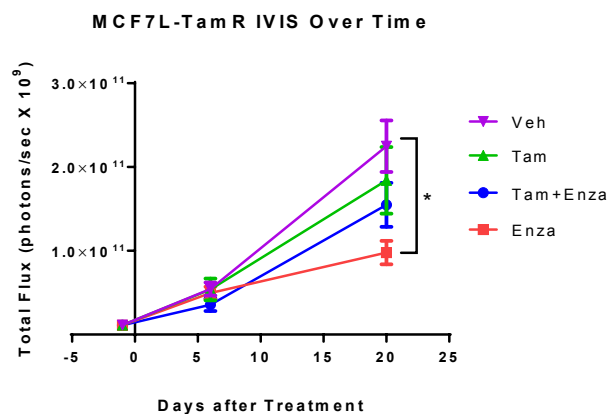
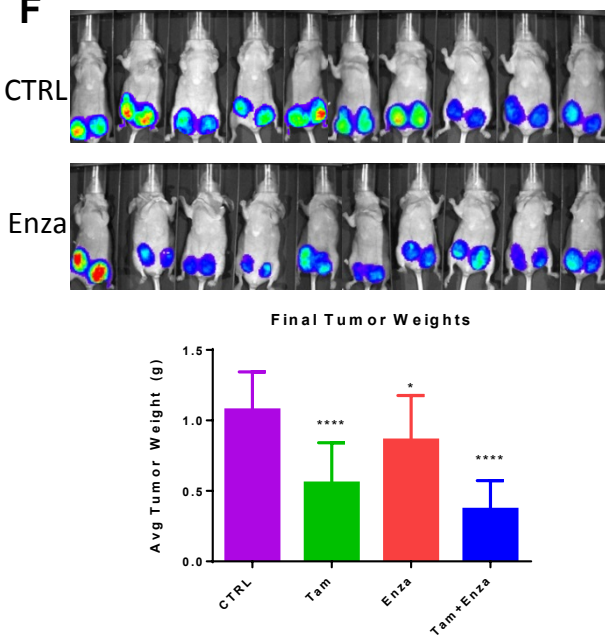
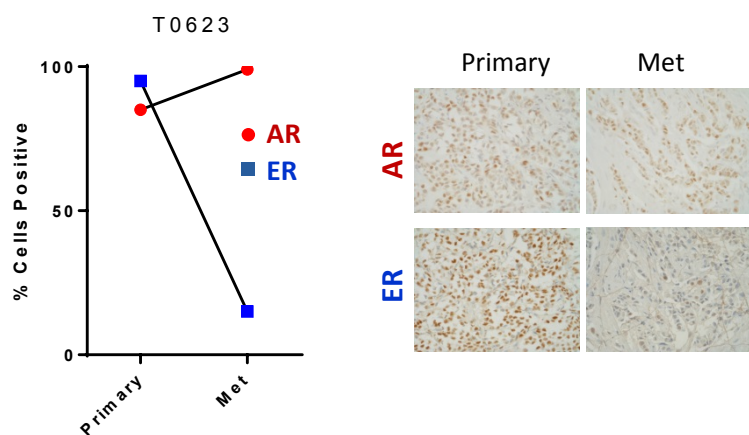


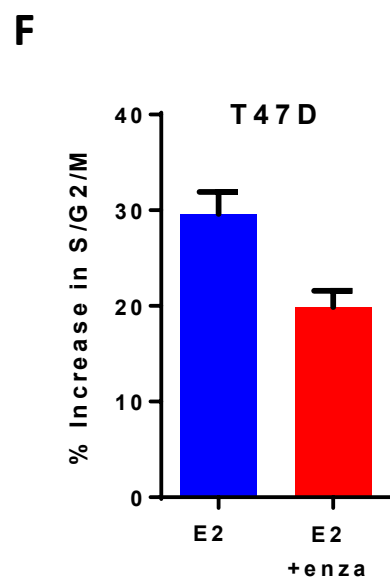
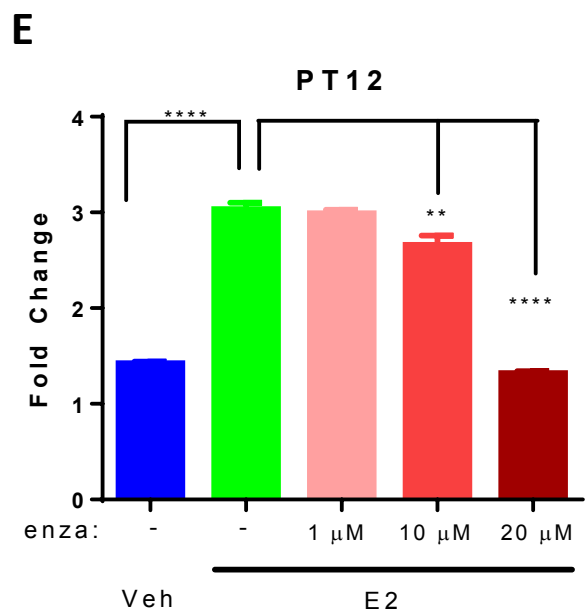
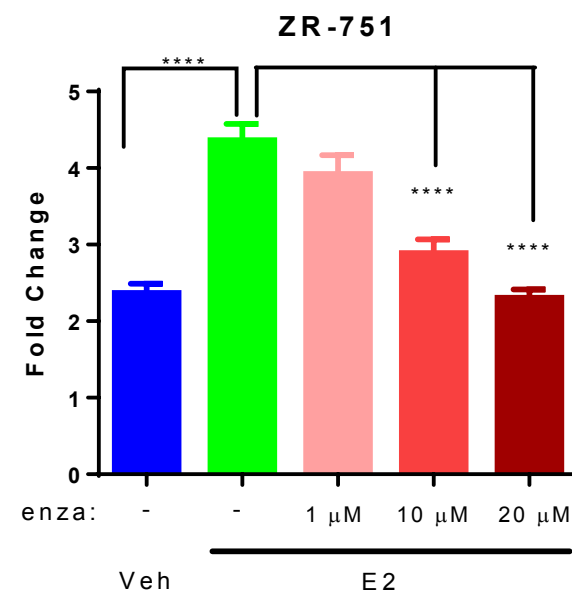
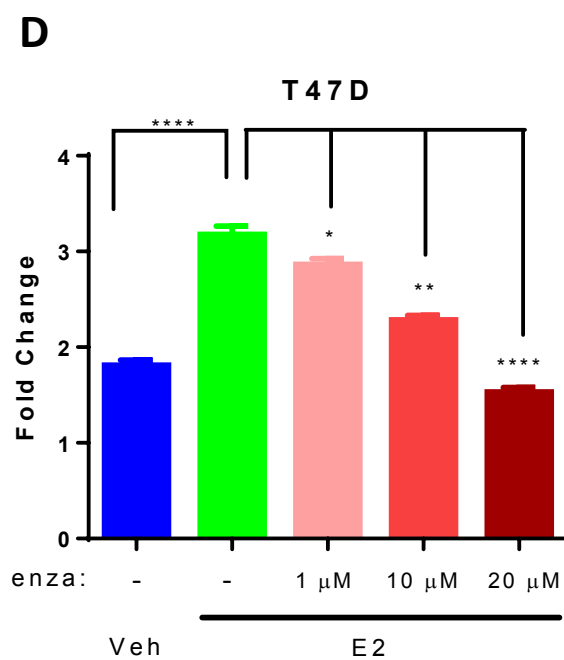
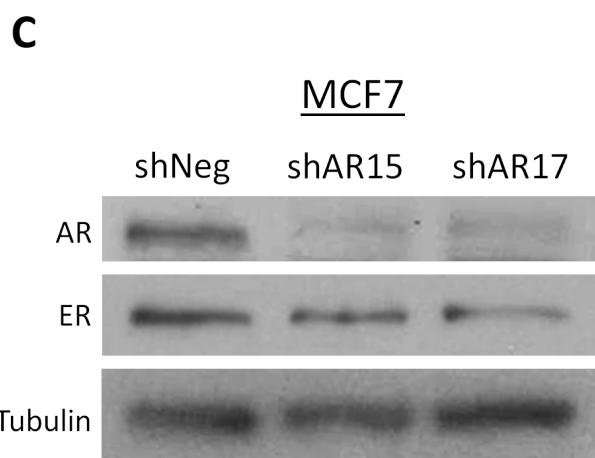
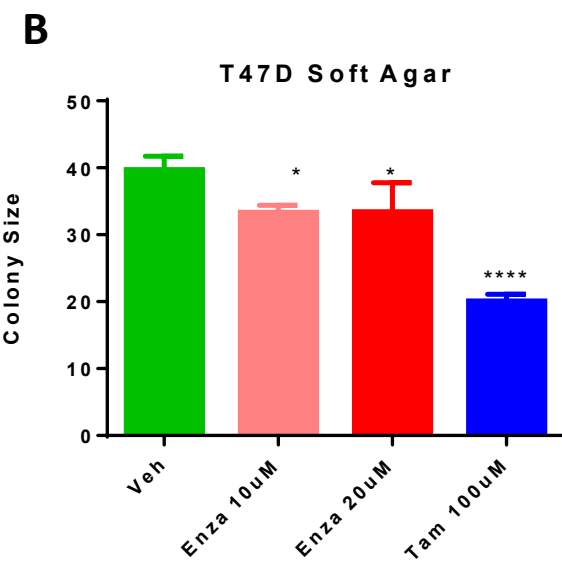
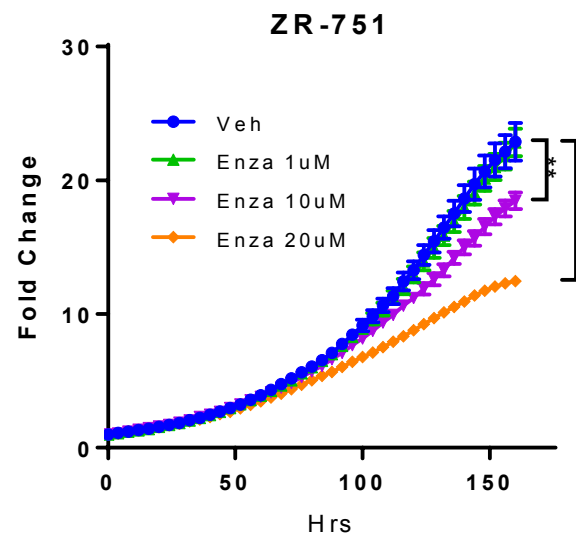
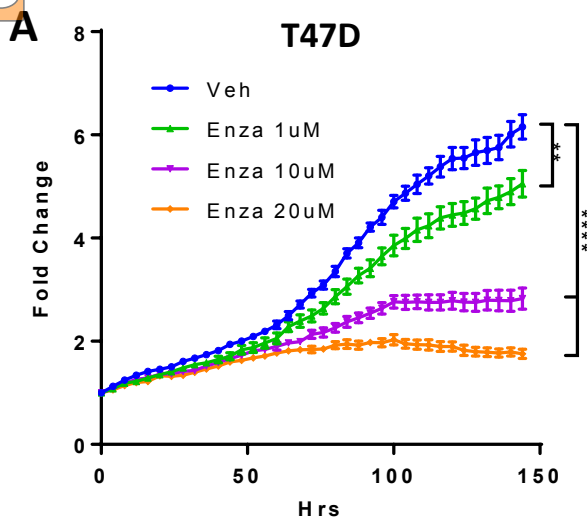
ER α staining

B

ER α IHC

Figure 5

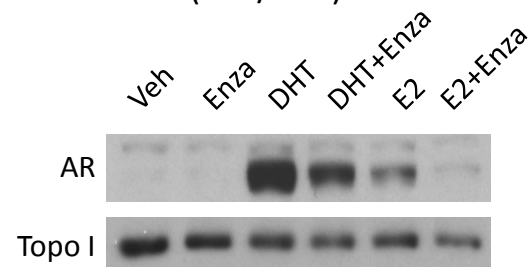
**C****D****E****F****G**



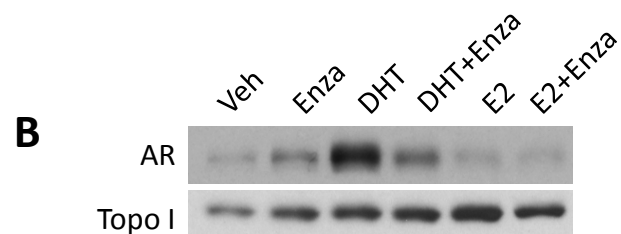
Extended Data Figure 1



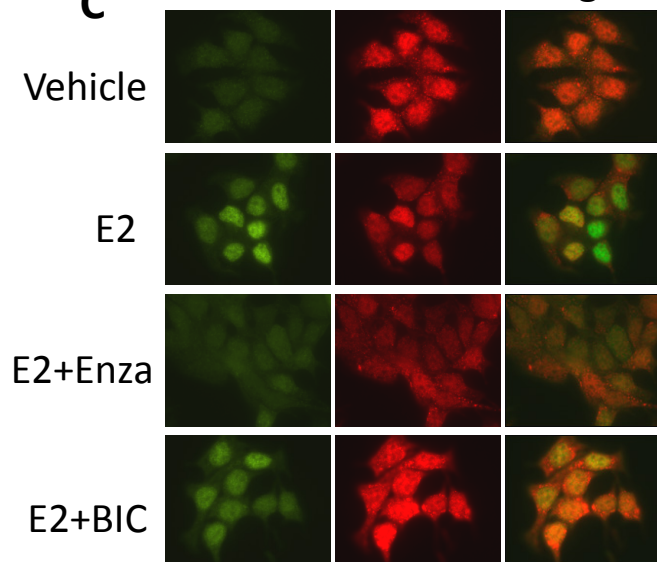
A ZR-75-1 (ER+/AR+) Nuclear Fraction



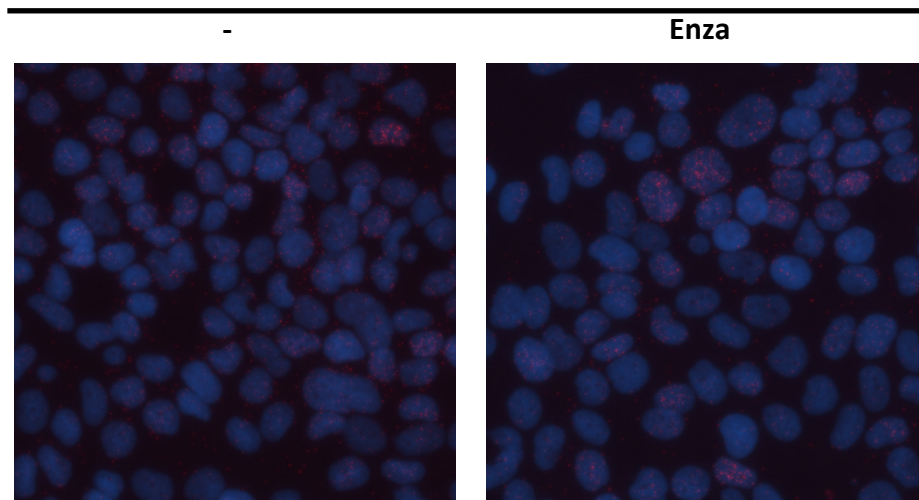
B MDA-453 (ER-/AR+) Nuclear Fraction



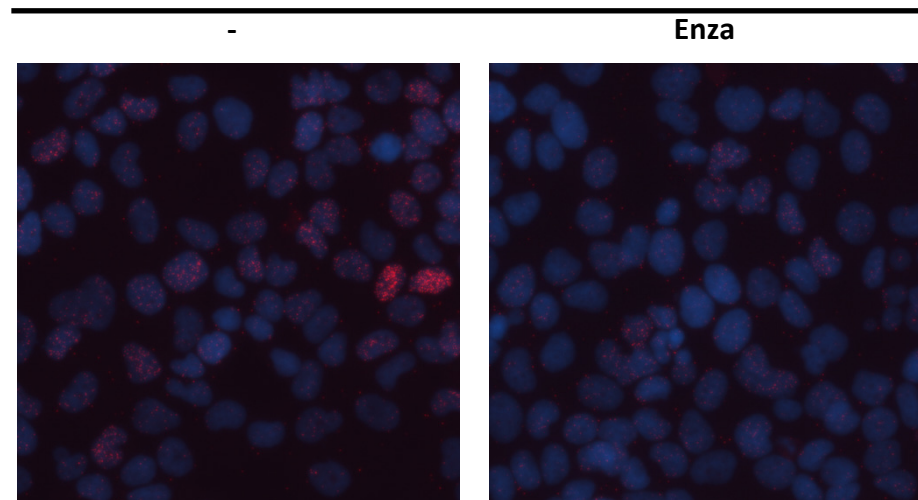
C AR ER Merge



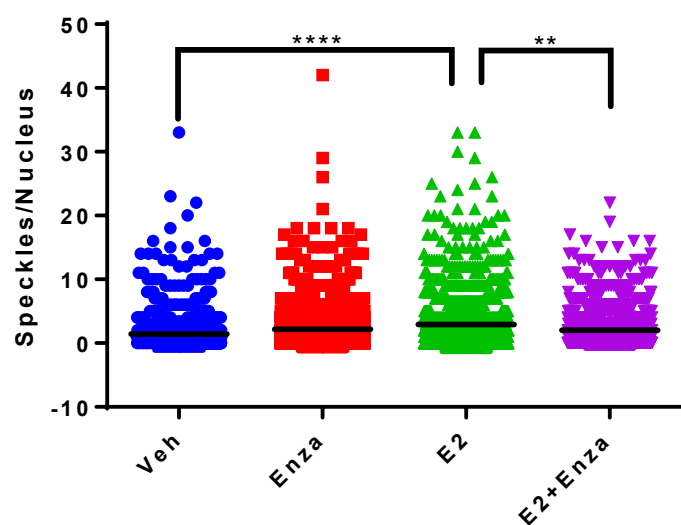
D Vehicle



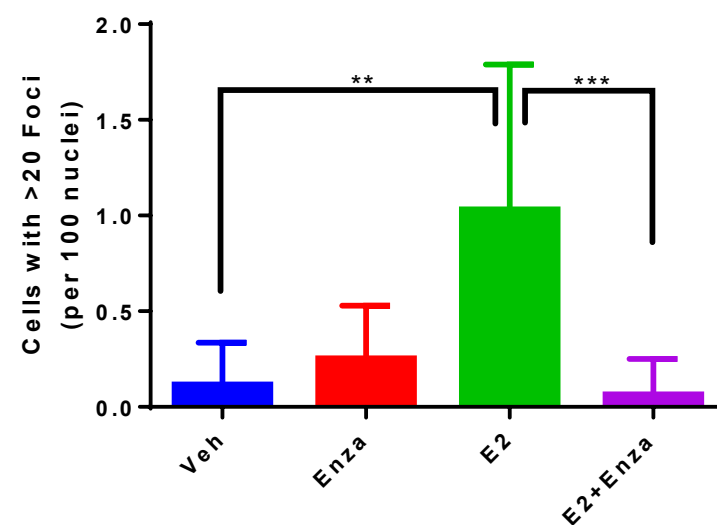
10nM E2 1hr



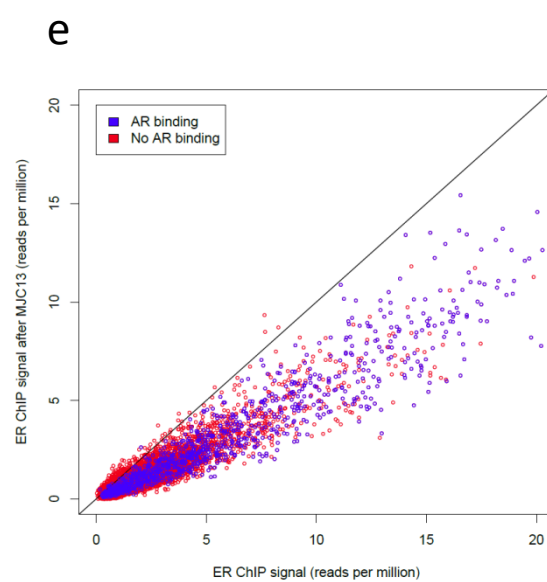
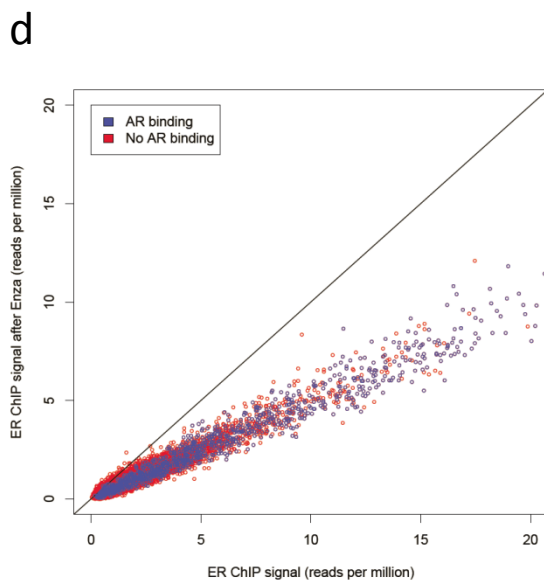
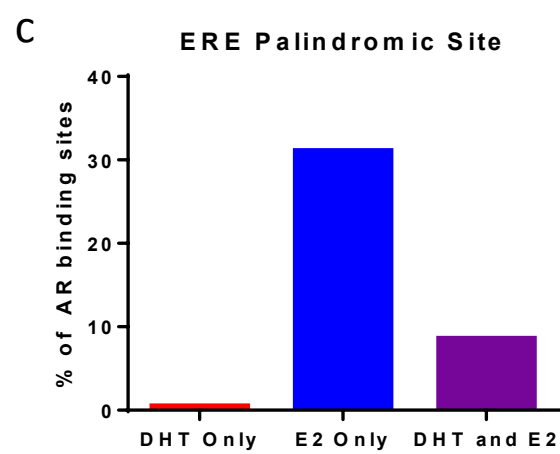
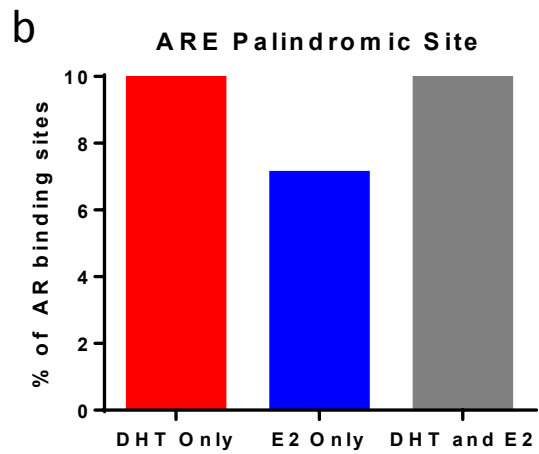
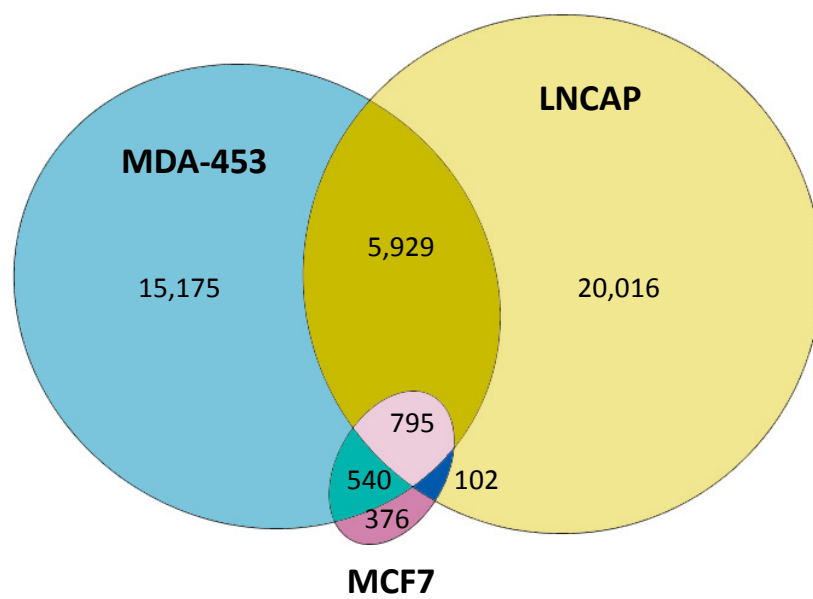
E ER/AR Interactions – Per Nucleus



F ER/AR Interactions – Per Nucleus



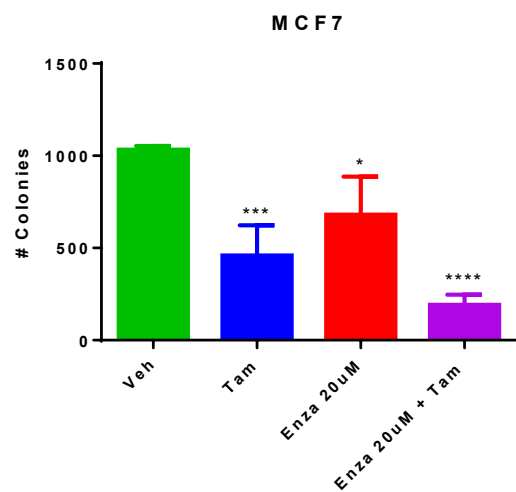
Extended Data Figure 2



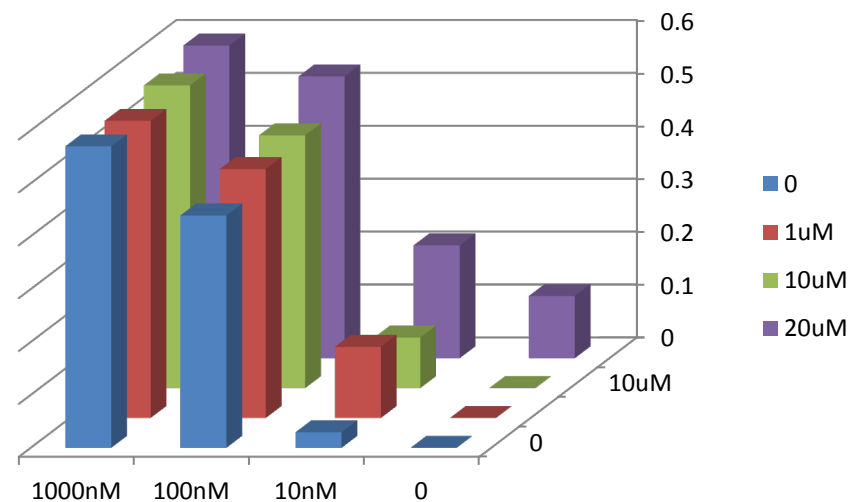
Extended Data Figure 3



a

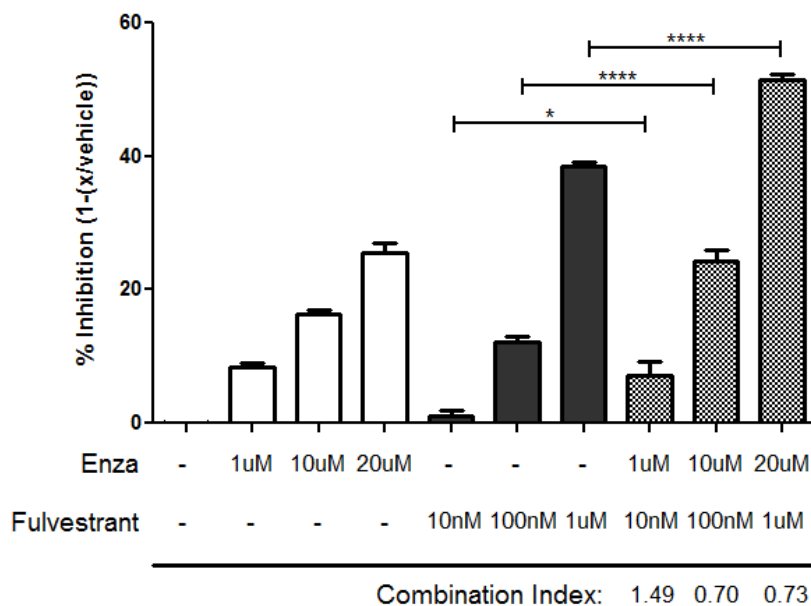
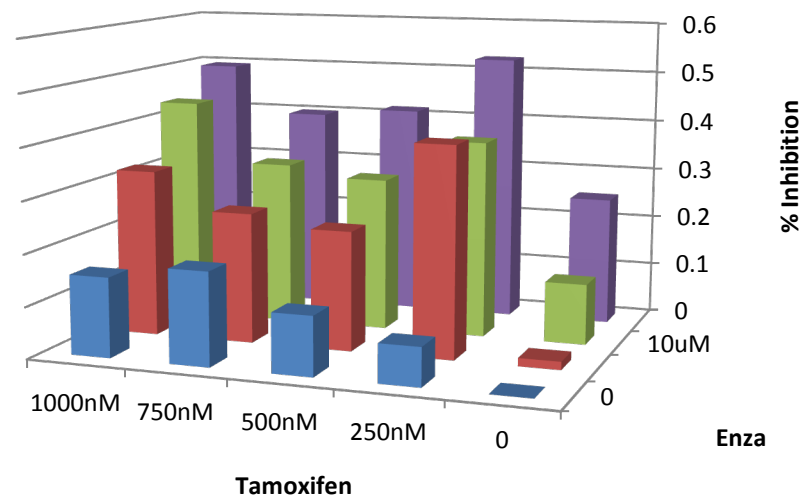


b

PT12 ICI / Enza Synergy

		Combination Index		
		ICI		
		10nM	100nM	1000nM
Enza	1uM	0.244	0.283	1.812
	10uM	0.53	0.275	1.721
	20uM	0.159	0.211	1.565

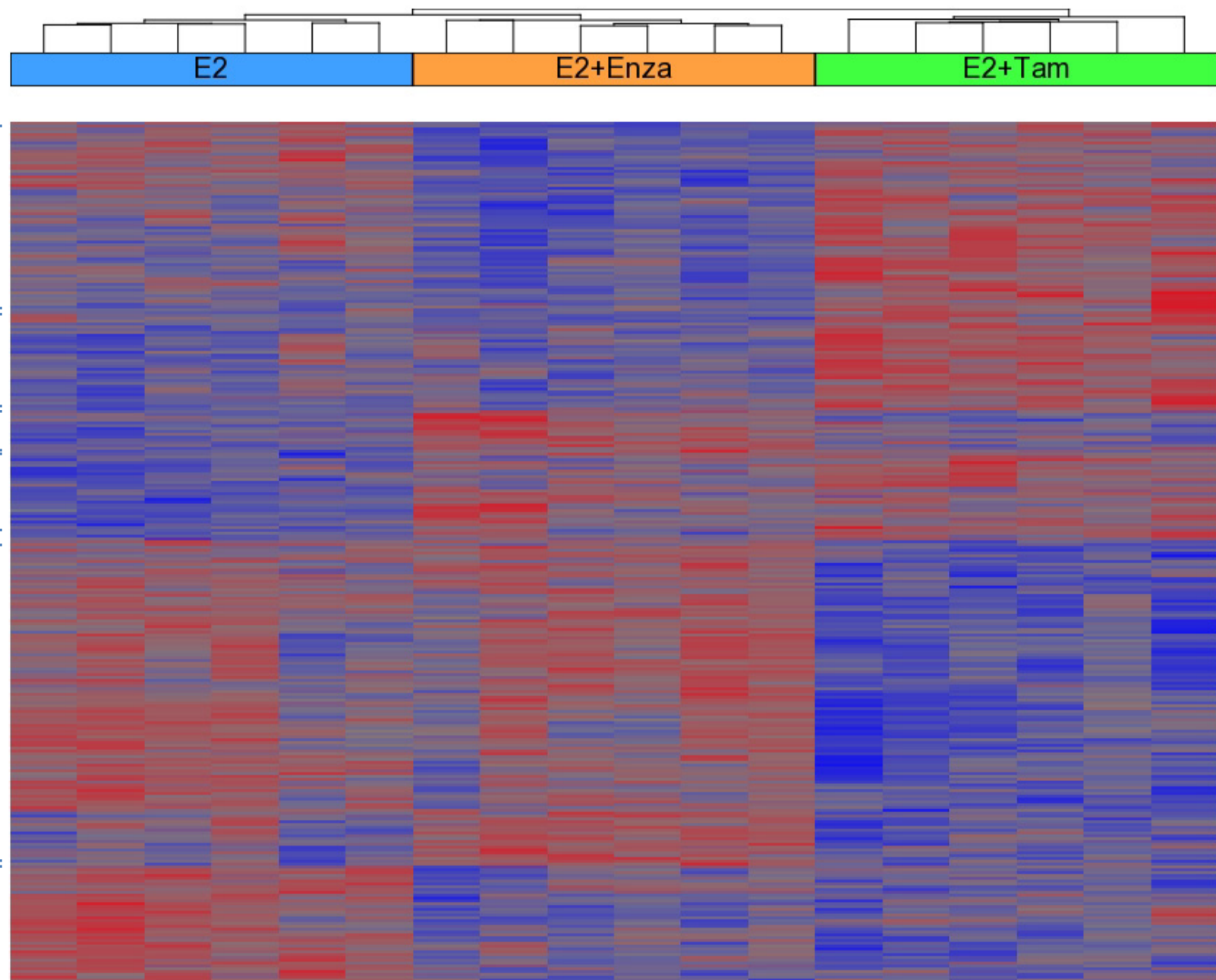
c

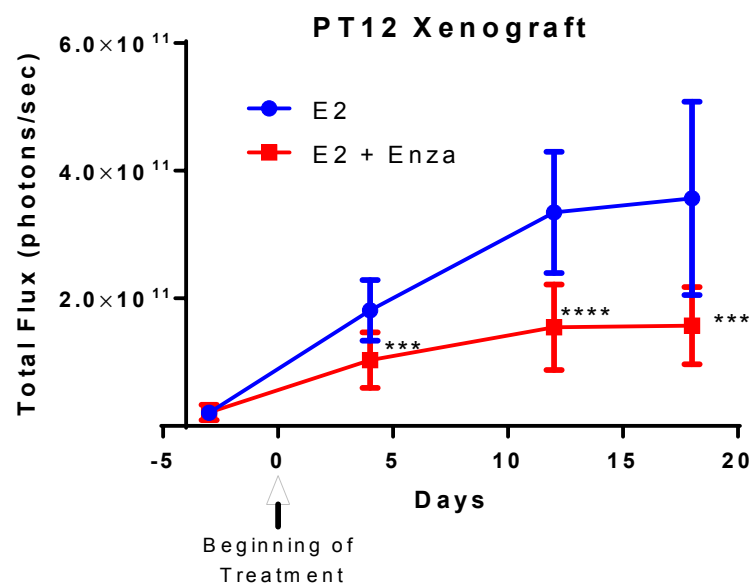
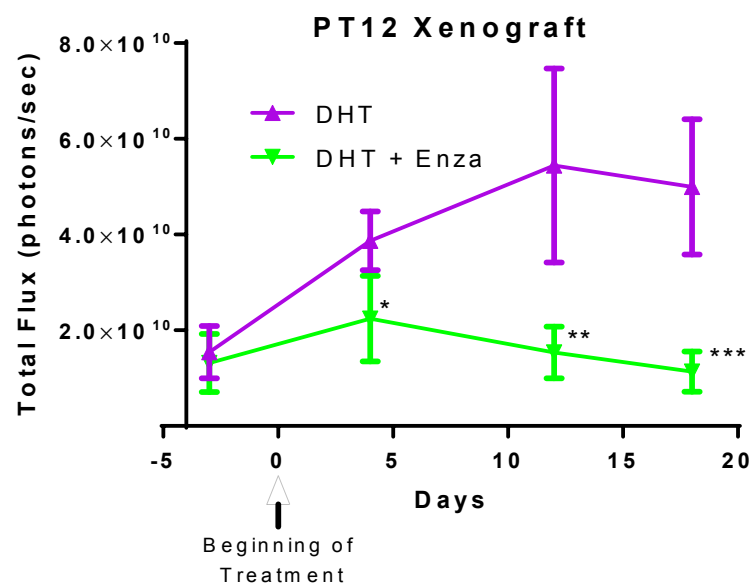
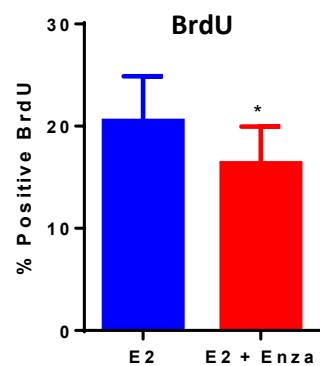
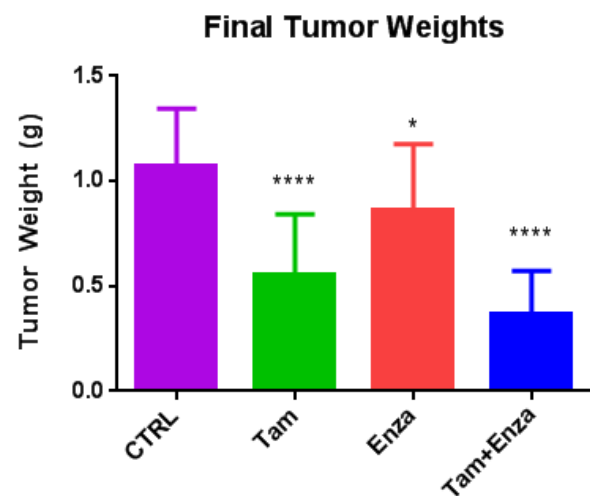
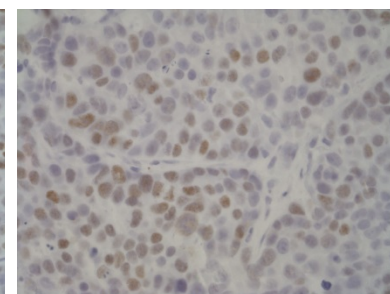
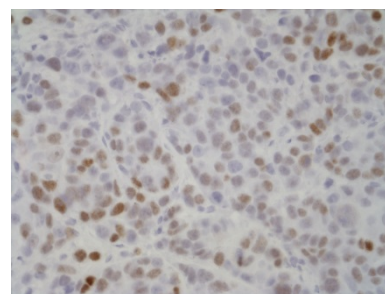
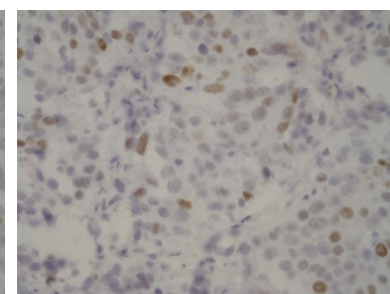
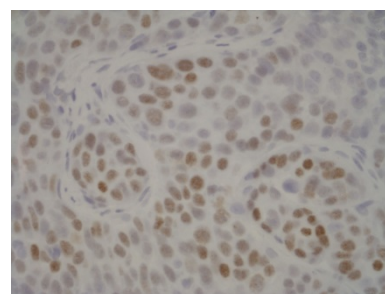
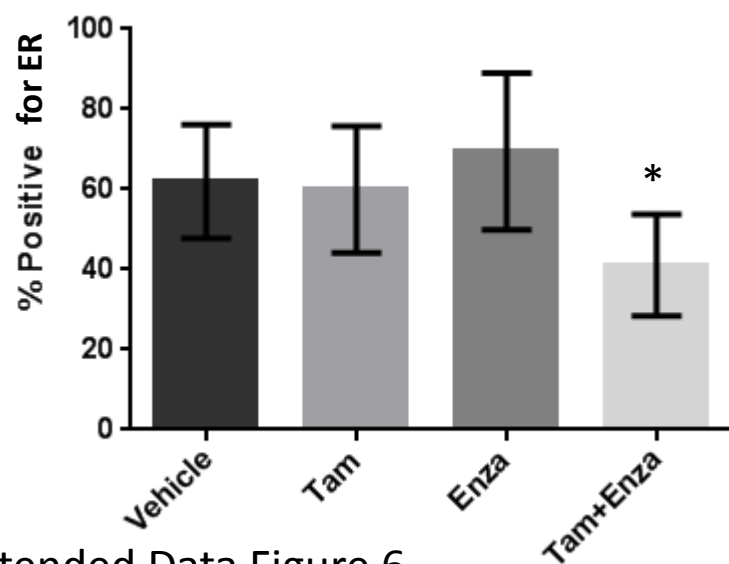
E2-Induced Proliferation in ZR-75-1 Cells**T47D Enza / Tam Baseline Synergy**

		Combination Index			
		Tamoxifen			
		250nM	500nM	750nM	1000nM
Enza	5uM	0.244	0.593	0.659	0.544
	10uM	0.466	0.675	0.710	0.529
	20uM	0.656	0.867	0.947	0.781

Extended Data Figure 4

A



**A****B****C****D****E****F****Veh****Tam****Enza****Tam+Enza****E**



Character limit: 3400 including title, body, tables and spaces
Character count: 3260
Subject Category: 633. Treatment - Other

MDV3100-08: A phase 1 study evaluating the safety and pharmacokinetics of enzalutamide plus fulvestrant in women with advanced hormone receptor-positive breast cancer.

Anthony D. Elias, Howard A. Burris, Manish R. Patel, Lee Steven Schwartzberg, Jennifer K. Richer, Edward Kavalierchik, Shanna Stopatschinskaja, Jackie Gibbons, Denka Markova, Joyce L. Steinberg, Tiffany A. Traina

University of Colorado Cancer Center, Aurora, CO; Sarah Cannon Research Institute, Nashville, TN; Florida Cancer Specialists, Sarasota, FL; The University of Tennessee Health Science Center, Memphis, TN; University of Colorado Cancer Center, Aurora, CO; Medivation, Inc., San Francisco, CA; Medivation, Inc., San Francisco, CA; Medivation, Inc., San Francisco, CA; Astellas Pharmaceuticals, Inc., Northbrook, IL; Memorial Sloan Kettering Cancer Center, New York, NY

Background: Fulvestrant (FUL), an estrogen receptor (ER) antagonist, is an effective treatment for patients (pts) with hormone receptor-positive (HR+) breast cancer (BC) whose disease has progressed or recurred during previous anti-estrogen therapy. The androgen receptor (AR), expressed in the majority of HR+ BC, may contribute to resistance to hormonal therapy. Enzalutamide (ENZA) is a potent inhibitor of AR signaling. Preclinical models with ER+/AR+ BC cell lines showed synergistic inhibitory effects for ENZA combined with FUL on tumor cell growth. ENZA is a potent CYP3A4 inducer, and in vitro studies show that CYP3A4 is the only CYP enzyme involved in the oxidative metabolism of FUL. In this phase 1 trial (NCT01597193), we evaluated the potential for ENZA to affect FUL pharmacokinetics (PK), as well as the safety and tolerability of the combination of ENZA with FUL.

Methods: Postmenopausal pts with HR+/AR+ advanced BC were enrolled; any number of prior therapies were permissible. Tumor tissue was analyzed centrally for AR expression; pts who had $\geq 10\%$ tumor cells with nuclear AR staining were eligible. All pts received at least 3 doses of FUL (500 mg intramuscularly on days 1, 15, and 29 and once monthly thereafter) to ensure steady-state concentrations prior to initiating ENZA 160 mg/day orally. The combination of ENZA with FUL was given until disease progression. PK and hormone sampling occurred on day 1 prior to ENZA initiation and on days 29 and 57. All pts were monitored for safety and response to treatment.

Results: As of 01May2015, 11 pts were enrolled; PK data are available for 8 of 11 pts, and 6 pts remain on study. Median age was 59 years; median ECOG performance status was 1. Two pts previously received FUL as a prior therapy for advanced BC; 4 pts received no prior therapy for advanced BC. The median duration of exposure to the combination was 16.6 weeks (range 4.0-42.3); the median duration of exposure to FUL (including at least 3 preloading doses) was 24.4 weeks (range 11.7-67.3). Common (>2 pts) ENZA-related adverse events (AEs) included fatigue (n=6), nausea (n=5), cognitive disorder (n=4) and diarrhea (n=3). Cognitive changes Grade 1/2 were reported in 4 pts based on the cognitive function assessment questionnaire. Two pts reported unrelated serious AEs (erosive gastritis, urinary tract

infection, iron deficiency anemia, and dehydration). Four pts had AEs \geq Grade 3: hypertension, anemia, hyperglycemia, urinary tract infection, asthenia, erosive gastritis, dehydration, and iron deficiency anemia; only asthenia and hypertension were considered treatment-related. Circulating levels of estradiol and estrone were within the expected range. Trough plasma concentrations of FUL (C_{min}) were similar for FUL alone and FUL combined with ENZA ($C_{min}=13.7 \pm 2.8$ and 12.5 ± 1.8 ng/mL, respectively).

Conclusions: The safety profile for the combination of daily ENZA with FUL is consistent with the published data for ENZA and FUL monotherapies. ENZA with FUL achieves similar plasma exposure to FUL alone, indicating no PK drug interaction.